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CHEMICAL ABSTRACTS, vol. 88, no. 15, 10th April 1978, page 249, no. 101458c Columbus, Ohio, U.S.A. K. BACKMAN et al.: "Maximizing gene expression on a plasmid using recombination in vitro"

NATURE, vol. 281, 18th October 1979, pages 544-548 D.V. GOEDDEL et al.: "Direct expression in Escherichia coli of a DNA sequence coding for human growth hormone"

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NATURE, vol. 275, 19th october 1978, pages 617-624 ANNIE C.Y. CHANG et al.: "Phenotypic expression in E. coli of a DNA sequence coding for mouse dihydrofolate reductase"

NATURE, vol. 263, 28th October 1976, pages 744-748 K.J. MARIANS et al.: "Cloned synthetic lac operator DNA is biologically active"

NATURE, vol. 276, 21st/28th December 1978, pages 795-798 P.H. SEEBURG et al.: "Synthesis of growth hormone by bacteria"

Description

Background of the Invention

Genetic Expression

The DNA (deoxyribonucleic acid) of which genes are made comprises both protein-encoding or "structural" genes and control regions that mediate the expression of their information through provision of sites for RNA polymerase binding, information for ribosomal binding sites, etc. Encoded protein is "expressed" from its corresponding DNA by a multistep process within an organism by which:

1. The enzyme RNA polymerase is activitated in the control region (hereafter the "promoter") and travels along the structural gene, transcribing its encoded information into messenger ribonucleic acid (mRNA) until transcription is ended at one or more "stop" codons.
2. The mRNA message is translated at the ribosomes into a protein for whose amino acid sequence the gene encodes, beginning at a translation "start" signal, most commonly ATG (which is translated "f-methionine").

In accordance with the genetic code, DNA specifies each amino acid by a triplet or "codon" of three adjacent nucleotides individually chosen from adenosine, thymidine, cytidine and guanine or, as used herein, A,T,C, or G. These appear in the coding strand or coding sequence of double-stranded ("duplex") DNA, whose remaining or "complementary" strand is formed of nucleotides ("bases") which hydrogen bond to their complements in the coding strand. A complements T, and C complements G. These and other subjects relating to the background of the invention are discussed at length in Benjamin Lewin, Gene Expression 1, 2 (1974) and 3 (1977), John Wiley and Sons, N.Y.

DNA Cleavage and Ligation

A variety of techniques are available for DNA recombination, according to which adjoining ends of separate DNA fragments are tailored in one way or another to facilitate ligation. The latter term refers to the formation of phosphodiester bonds between adjoining nucleotides, most often through the agency of the enzyme T4 DNA ligase. Thus, blunt ends may be directly ligated. Alternatively, fragments containing complementary single strands at their adjoining ends are advantaged by hydrogen bonding which positions the respective ends for subsequent ligation. Such single strands, referred to as cohesive termini, may be formed by the addition of nucleotides to blunt ends using terminal transferase, and sometimes simply by chewing back one strand of a blunt end with an enzyme such λ -exonuclease. Again, and most commonly, resort may be had to restriction endonucleases (hereafter, "restriction enzymes"), which cleave phosphodiester bonds in and around unique sequences of nucleotides of about 4-6 base pairs in length ("restriction sites"). Many restriction enzymes and their recognition sites are known. See, e.g., R. J. Roberts, CRC Critical Reviews in Biochemistry, 123 (Nov. 1976). Many make staggered cuts that generate short complementary single-stranded sequences at the ends of the duplex fragments. As complementary sequences, the protruding or "cohesive" ends can recombine by base pairing. When two different molecules are cleaved with this enzyme, crosswise pairing of the complementary single strands generates a new DNA molecule, which can be given covalent integrity by using ligase to seal the single strand breaks that remain at the point of annealing. Restriction enzymes which leave coterminal or "blunt" ends on duplex DNA that has been cleaved permit recombination via, e.g., T4 ligase with other blunt-ended sequences.

Cloning Vehicles and Recombinant DNA

For present purposes, a "cloning vehicle" is a nonchromosomal length of duplex DNA comprising an intact replicon such that the vehicle can be replicated when placed within a unicellular organism ("microbe") by transformation. An organism so transformed is called a "transformant". Presently, the cloning vehicles commonly in use are derived from viruses and bacteria and most commonly are loops of bacteria DNA called "plasmids".

Advances in biochemistry in recent years have led to the construction of "recombinant" cloning vehicles in which, for example, plasmids are made to contain exogenous DNA. In particular instances the recombinant may include "heterologous" DNA, by which is meant DNA that codes for polypeptides ordinarily not produced by the organism susceptible to transformation by the recombinant vehicle. Thus, plasmids are cleaved with restriction enzymes to provide linear DNA having ligatable termini. These are bound to an exogenous gene having ligatable termini to provide a biologically functional moiety with an

intact replicon and a phenotypical property useful in selecting transformants. The recombinant moiety is inserted into a microorganism by transformation and the transformant is isolated and cloned, with the object of obtaining large populations that include copies of the exogenous gene and, in particular cases, with the further object of expressing the protein for which the gene codes. The associated technology and its potential applications are reviewed in extenso in the Miles International Symposium Series 10: Recombinant Molecules: Impact on Science and Society, Beers and Bosseff, eds., Raven Press, N.Y. (1977).

Recombinant DNA Expression

Aside from the use of cloning vehicles to increase the supply of genes by replication, there have been attempts, some successful, to actually express proteins for which the genes code. In the first such instance a gene for the brain hormone somatostatin under the influence of the lac promotor was expressed in E. Coli bacteria. K. Itakura et al, Science 198, 1056 (1977). More recently, the A and B chains of human insulin were expressed in the same fashion and combined to form the hormone. D. V. Goeddel et al., Proc. Nat'l. Acad. Sci., USA 76, 106 (1979). In each case the genes were constructed in their entirety by synthesis. In each case, proteolytic enzymes within the cell would apparently degrade the desired product, necessitating its production in conjugated form, i.e., in tandem with another protein which protected it by compartmentalization and which could be extracellulary cleaved away to yield the product intended. This work is described in the following published British patent specifications of the assignee of the present application: GB 2007 675 A; GB 2 007 670 A; GB 2 007 676 A; and GB 2 008 123 A.

While the synthetic gene approach has proven useful in the several cases thus far discussed, real difficulties arise in the case of far larger protein products, e.g., growth hormone or interferon, whose genes are correspondingly more complex and less susceptible to facile synthesis. At the same time, it would be desirable to express such products unaccompanied by conjugate protein, the necessity of whose expression requires diversion of resources within the organism better committed to construction of the intended product.

Other workers have attempted to express genes derived not by organic synthesis but rather by reverse transcription from the corresponding messenger RNA purified from tissue. Two problems have attended this approach. To begin with, reverse transcriptase may stop transcription from mRNA short of completing cDNA for the entire amino acid sequence desired. Thus, for example, Villa-Komaroff et al obtained cDNA for rat proinsulin which lacked codons for the first three amino acids of the insulin precursor. Proc. Nat'l. Acad. Sci., USA 75 3727 (1978). Again, reverse transcription of mRNA for polypeptides that are expressed in precursor form has yielded cDNA for the precursor form rather than the bioactive protein that results when, in a eukaryotic cell, leader sequences are enzymatically removed. Thus far, no bacterial cell has been shown to share that capability, so that mRNA transcripts have yielded expression products containing the leader sequences of the precursor form rather than the bioactive protein itself. Villa-Komaroff, supra (rat proinsulin); P. H. Seeburg et al, Nature 276, 795 (1978) (rat pregrowth hormone).

Finally, past attempts by others to bacterially express human hormones (or their precursors) from mRNA transcripts have on occasion led only to the production of conjugated proteins not apparently amenable to extra-cellular cleavage, e.g., Villa-Komaroff, supra, (penicillinase-proinsulin); Seeburg, supra (beta-lactamase-pregrowth hormone).

Human Growth Hormone

Human growth hormone ("HGH") is secreted in the human pituitary. It consists of 191 amino acids and, with its molecular weight of about 21,500, is more than three times as large as insulin. Until the present invention, human growth hormone could be obtained only by laborious extraction from a limited source — the pituitary glands of human cadavers. The consequent scarcity of the substance has limited its applications to the treatment of hypopituitary dwarfism, and even here reliable estimates suggest that human-derived HGH is available in sufficient quantity to serve not more than about 50% of afflicted subjects.

In summary, a need has existed for new methods of producing HGH and other polypeptide products in quantity, and that need has been particularly acute in the case of polypeptides too large to admit of organic synthesis or, for that matter, microbial expression from entirely synthetic genes. Expression of mammalian hormones from mRNA transcripts has offered the promise of sidestepping difficulties that attend the synthetic approach, but until the present has permitted only microbial production of bio-inactive conjugates from which the desired hormone could not practicably be cleaved.

Summary of the Invention

The present invention provides methods and means for expressing quasi-synthetic genes wherein reverse transcription provides a substantial portion, preferably a majority, of the coding sequence without laborious resort to entirely synthetic construction, while synthesis of the remainder of the coding sequence affords a completed gene capable of expressing the desired polypeptide unaccompanied by bio-inactivating leader sequences or other extraneous protein. Alternatively, the synthetic remainder may yield a proteolysis-resistant conjugate so engineered as to permit extra-cellular cleavage of extraneous protein, yielding the bioactive form. The invention accordingly makes available methods and means for microbial production of numerous materials hitherto produced only in limited quantity by costly extraction from tissue, and still others previously incapable of industrial manufacture. In its most preferred embodiment the invention represents the first occasion in which a medically significant polypeptide hormone (human growth hormone) has been bacterially expressed while avoiding both intracellular proteolysis and the necessity of compartmentalizing the bioactive form in extraneous protein pending extracellular cleavage. Microbial sources for human growth hormone made available by the invention offer, for the first time, ample supplies of the hormone for treatment of hypopituitary dwarfism, together with other applications heretofore beyond the capacity of tissue-derived hormone sources, including diffuse gastric bleeding, pseudarthrosis, burn therapy, wound healing, dystrophy and bone knitting.

The manner in which these and other objects and advantages of the invention may be obtained will appear more fully from the detailed description which follows, and from the accompanying drawings relating to a preferred embodiment of the invention, in which:

Figure 1 depicts the synthetic scheme for construction of a gene fragment coding for the first 24 amino acids of human growth hormone, together with the start signal ATG and linkers used in cloning. The arrows in the coding or upper strand ("U") and in the complementary or lower strand ("L") indicate the oligonucleotides joined to form the depicted fragment;

Figure 2 depicts joinder of the "U" and "L" oligonucleotides to form the gene fragment of Figure 1, and its insertion in a plasmid cloning vehicle;

Figure 3 illustrates the DNA sequence (coding strand only) of the Hae III restriction enzyme fragment of a pituitary mRNA transcript, with the numbered amino acids of human growth hormone for which they code. Key restriction sites are indicated, as is DNA (following "stop") for untranslated mRNA;

Figure 4 illustrates the construction of a cloning vehicle for a gene fragment coding for the amino acids of human growth hormone not synthetically derived, and the construction of that gene fragment as complementary DNA by reverse transcription from mRNA isolated from a human pituitary source; and

Figure 5 illustrates the construction of a plasmid capable, in bacteria, of expressing human growth hormone, beginning with the plasmids of Figures 2 and 3.

Detailed Description of the Invention

The general approach of the invention involves the combination in a single cloning vehicle of plural gene fragments which in combination code for expression of the desired product. Of these, at least one is a cDNA fragment derived by reverse transcription from mRNA isolated from tissue, as by the method of A. Ullrich et al, Science 196, 1313 (1977). The cDNA provides a substantial portion, and preferably at least a majority, of the codons for the desired product, while remaining portions of the gene are supplied synthetically. The synthetic and mRNA transcript fragments are cloned separately to provide ample quantities for use in the later combination step.

A variety of considerations influence distribution of codons for the end product as between synthetic and cDNA, most particularly the DNA sequence of complementary DNA determined as by the method of Maxam and Gilbert, Proc. Nat'l Acad. Sci. USA 74, 560 (1977). Complementary DNA obtained by reverse transcription will invariably contain codons for at least a carboxy terminal portion of the desired product, as well as other codons for untranslated mRNA downstream from the translation stop signal(s) adjacent the carboxy terminus. The presence of DNA for untranslated RNA is largely irrelevant, although unduly lengthy sequences of that kind may be removed, as by restriction enzyme cleavage, to conserve cellular resources employed in replicating and expressing the DNA for the intended product. In particular cases, the cDNA will contain codons for the entire amino acid sequence desired, as well as extraneous codons upstream from the amino terminus of the intended product. For example, many if not all polypeptide hormones are expressed in precursor form with leader or signal sequences of protein involved, e.g., in transport to the cellular membrane. In expression from eukaryotic cells, these sequences are enzymatically removed, such that the hormone enters the periplasmic space in its free, bioactive form. However, microbial cells cannot be

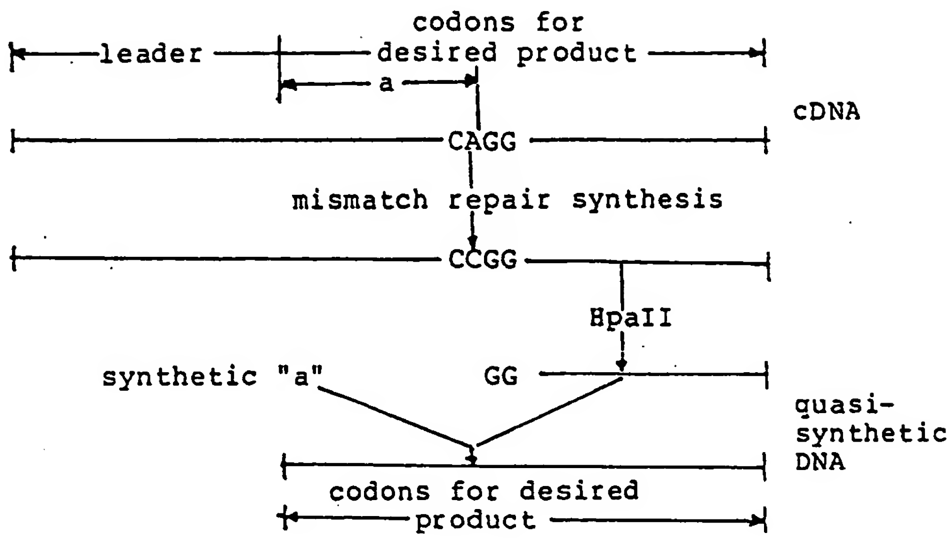
relied upon to perform that function, and it is accordingly desirable to remove sequences coding for such signals or leader sequences from the mRNA transcript. In the course of that removal process the translation start signal is also lost, and almost invariably some codons for the intended product will be removed as well. The synthetic component of the quasi-synthetic gene product of the invention returns these latter codons, as well as supplying anew a translation start signal where the vehicle into which the hybrid gene will ultimately be deployed itself lacks a properly positioned start.

Elimination of the leader sequence from pregrowth hormone cDNA is advantaged by the availability of a restriction site within the growth hormone-encoding portion of the gene. The invention may nevertheless be practiced without regard to the availability of such a site, or in any event without regard to the availability of a restriction site sufficiently near the amino terminus of the desired polypeptide as to obviate the need for extensive synthesis of the gene component not derived from mRNA. Thus, in any cDNA coding for the desired polypeptide and a leader or other bioinactivating sequence the boundary between the latter's codons and those of the mature polypeptide will appear from the amino acid sequence of the mature polypeptide. One may simply digest into the gene coding from the peptide of choice, removing the unwanted leader or other sequence. Thus, for example, given cDNA such as:

```
        |<-a->|<-b-->|
5'      |     |      |
        TTAAGCCCTGATCGT ...
                              etc.
        AATTCGGGACTAGCA ...
3'      |     |      |
        |<-c->|<-d-->|
```

where the endpoint of digestion is indicated by arrow, reaction conditions for exonuclease digestion may be chosen to remove the upper sequences "a' and "b", whereafter S1 nuclease digestion will automatically eliminate the lower sequences "c" and "d". Alternatively and more precisely, one may employ DNA polymerase digestion in the presence of deoxynucleotide triphosphates ("d(A,T,C,G)TP"). Thus, in the foregoing example, DNA polymerase in the presence of dGTP will remove sequence "c" (then stop at "G"), S1 nuclease will then digest "a"; DNA polymerase in the presence of dTTP will remove "d", (then stop at "T") and S1 nuclease will then excise "b", and so on. See generally A. Kornberg, DNA Synthesis, pp. 87-88, W. H. Freeman and Co., San Francisco (1974).

More preferably, one may simply construct a restriction site at a convenient point within the portion of the cDNA coding for the desired product, by an application of the mismatch repair synthesis technique of A. Razin et al, Proc. Nat'l Acad. Sci. USA 75, 4268 (1978). By this technique one or more bases may be substituted in an existing DNA sequence, using primers containing the mismatched substituent. At least seven palindromic 4-base pair sequences are uniquely recognized by known restriction enzymes, i.e., AGCT (Alu I), CCGG (Hpa II), CGCG (Tha I), GATC (Sau 3A), GCGC (Hha), GGCC (Hae III), and TCGA (Taq I). Where the cDNA sequence contains a sequence differing from one such site in a single base, as statistically is highly likely, repair synthesis will yield replicate cDNA containing the proper, substituent base and hence the desired restriction site. Cleavage will delete DNA for the unwanted leader, after which synthesis will replace codons required for expression of the complete polypeptide. E.g.,:



It will be appreciated, of course, that longer restriction sites may be likewise inserted where desired, or that successive repairs may create 4-base pair restriction sites where only two bases common to the site appear at the desired point, etc.

Applications will appear in which it is desirable to express not only the amino acid sequence of the intended product, but also a measure of extraneous but specifically engineered protein. Four such applications may be mentioned by way of example. First, the quasi-synthetic gene may represent a hapten or other immunological determinant upon which immunogenicity is conferred by conjugation to additional protein, such that vaccines are produced. See generally, G.B. patent specification 2 008 123A. Again, it may be desirable for biosafety reasons to express the intended product as a conjugate other, bio-inactivating protein so designed as to permit extracellular cleavage to yield the active form. Third, applications will be presented in which transport signal polypeptides will precede the desired product, to permit production of the same by excretion through the cell membrane, so long as the signal peptide can then be cleaved. Finally, extraneous conjugate designed to permit specific cleavage extracellularly may be employed to compartmentalize intended products otherwise susceptible to degradation by proteases endogenous to the microbial host. At least in the latter three applications, the synthetic adaptor molecular employed to complete the coding sequence of the mRNA transcript can additionally incorporate codons for amino acid sequences specifically cleavable, as by enzymatic action. For example, trypsin or chymotrypsin will cleave specifically at arg-arg or lys-lys, etc. See GB 2 008 123A, supra.

From the foregoing, it will be seen that in its broadest aspect the invention admits of manifold applications, each having in common these attributes:

- a mRNA transcript is employed which codes for a substantial portion of the intended polypeptide's amino acid sequence but which, if expressed alone, would produce a different polypeptide either smaller or larger than the intended product;
- protein-encoding codons for amino acid sequences other than those contained in the intended product, if any, are removed;
- organic synthesis yields fragment(s) coding for the remainder of the desired sequence; and
- the mRNA transcript and synthetic fragment(s) are combined and disposed in a promoter containing cloning vehicle for replication and expression of either the intended product absent extraneous conjugated protein, or intended product conjugated to but specifically cleavable from extraneous protein.

Of course, the expression product will in every case commence with the amino acid coded for by the translation start signal (in the case of ATG, f-methionine). One can expect this to be removed intracellularly, or in any event to leave the bioactivity of the ultimate product essentially unaffected.

Although it provides a method of general applicability in the production of useful proteins, including e.g. antibodies and enzymes, the invention is particularly suited to the expression of mammalian polypeptide hormones and other substances having medical applications, e.g., glucagon, gastrointestinal inhibitory polypeptide, pancreatic polypeptide, adrenocorticotropin, beta-endorphins, interferon, urokinase, blood clotting factors, and human albumin. A preferred embodiment illustrative of the invention is next discussed, in

which a quasi-synthetic gene coding for human growth hormone is constructed, cloned and microbially expressed.

Construction and Expression of a Cloning Vehicle for Human Growth Hormone

1. Cloning the Hae III fragment of the mRNA transcript (Figs. 3 and 4)

Polyadenylated mRNA for human growth hormone (HGH) was prepared from pituitary growth hormone-producing tumors by the procedure of A. Ullrich et al. Science 196, 1313 (1977) 1.5 µg of double strand ("ds") cDNA was prepared from 5 µg of this RNA essentially as described by Wickens et al. J. Biol Chem. 253 2483 (1978), except that RNA polymerase "Klenow fragment", H. Klenow, Proc. Nat'l. Aci. USA. 65, 168 (1970), was substituted for DNA Polymerase I in the second strand synthesis. The restriction pattern of HGH is such that Hae III restriction sites are present in the 3' noncoding region and in the sequence coding for amino acids 23 and 24 of HGH, as shown in Fig. 3. Treatment of ds HGH cDNA with Hae III gives a DNA fragment of 551 base pairs ("bp") coding for amino acids 24-191 of HGH. Thus, 90 ng of the cDNA was treated with Hae III, electrophoresed on an 8% polyacryclamide gel, and the region at 350 bp eluted. Approximately 1 ng of cDNA was obtained.

pBR322 prepared as in F. Bolivar et al., Gene 2 (1977) 95-113 was chosen as the cloning vehicle for the cDNA. pBR322 has been fully characterized, J.G. Sutcliffe, Cold Spring Harbor Symposium 43, 70 (1978) is a multicopy replicating plasmid which exhibits both ampicillin and tetracycline resistance owing to its inclusion of the corresponding genes ("Ap^R" and "Tc^R", respectively, in Fig. 4), and which contains recognition sites for the restriction enzymes Pst I, EcoRI and Hind III as shown in the Figure.

Cleavage products of both Hae III and Pst I are blunt ended. The GC tailing method of Chang. A.C.Y. et al. Nature 275 617 (1978) could accordingly be employed to combine the blunt-ended products of Pst I cleavage of pBR322 and of Hae III digestion of the mRNA transcript, inserting the cDNA fragment into the Pst I site of pBR322 in such manner as to restore the Hae III restriction sites (GG↓CC) on the cDNA while restoring the Pst I restriction sites (CTGCA↓G) at each end of the insert.

Thus, terminal deoxynucleotidyl transferase (TdT) was used to add approximately 20 dC residues per 3' terminus as described previously, Chang, A.Y.C., supra. 60 ng of Pst I-treated pBR322 was tailed similarly with about 10 dG residues per 3' terminus. Annealing of the dC-tailed ds cDNA with the dG-tailed vector DNA was performed in 130 µl of 10mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.25 mM EDTA. The mixture was heated to 70°C, allowed to cool slowly to 37°C (12 hours), then to 20°C (6 hours) before being used to transform E. Coli. x1776. DNA sequence analysis of the plasmid pHGH31 cloned in x1776 by the method of Maxam and Gilbert, Proc. Nat'l. Acad.Sci. USA 74, 560 (1977) resulted in confirmation of the codons for amino acids 24-191 of HGH, as shown in Figure 3.

E. Coli K-12 strain x1776 has the genotype F^- tonA53 dapD8 minA1 supE42 Δ40[gal-uvrB] λ^- minB2 rfb-2 nalA25 oms-2 thyA57* metC65 oms-1 Δ29[bioH-asd] cycB2 cycA1 hsdR2. x1776 has been certified by the National Institutes of Health as an EK2 host vector system.

x1776 has an obligate requirement for diaminopimelic acid (DAP) and cannot synthesize the mucopolysaccharide colanic acid. It thus undergoes DAP-less death in all environments where DAP is limiting but sufficient nutrients exist to support cellular metabolism and growth. It requires thymine or thymidine and undergoes thymineless death with degradation of DNA when thymine and thymidine are absent from the environment but when sufficient nutrients are present to sustain metabolic activity. x1776 is extremely sensitive to bile and thus is unable to survive and thus is unable to survive passage through the intestinal tract of rats. x1776 is extremely sensitive to detergents, antibiotics, drugs and chemicals. x1776 is unable to carry out either dark or photo repair of UV-induced damage and is thus several orders of magnitude more sensitive to sunlight than wild-type strains of E. Coli. x1776 is resistant to many transducing phages and is conjugation deficient for inheritance of many different types of conjugative plasmids due to the presence of various mutations. x1776 is resistant to nalidixic acid, cycloserine and trimethoprim. These drugs can therefore be added to media to permit monitoring of the strain and to preclude transformation of contaminants during transformation.

x1776 grows with a generation time of about 50 min. in either L broth or Penassay broth when supplemented with 100 µg DAP/ml and 4 µg thymidine/ml and reaches final densities of 8-10 x 10^8 cells/ml at stationary phase. Gentle agitation by swirling and shaking back and forth for a period of 1-2 min. adequately suspends cells with maintenance of 100% viability. Additional details concerning x1776 appear in R. Curtis et al., Molecular Cloning of Recombinant DNA, 99-177, Scott and Werner, eds., Academic Press (N.Y.1977). x1776 has been deposited in the American Type Culture Collection (July 3, 1979: ATCC accession no. 31537, without restriction.

2. Construction and Cloning of the Synthetic Gene Fragment (Figs. 1 and 2)

The strategy for construction of the HGH quasi-synthetic gene included construction of a synthetic fragment comprising a blunt-end restriction cleavage site adjacent the point at which the fragment would be joined to the mRNA transcript. Thus, as shown in Fig. 1, the synthetic gene for the first 24 amino acids of HGH contained a Hae III cleavage site following amino acid 23. The distal end of the synthetic fragment was provided with a "linker" that permitted annealing to a single strand terminal resulting from restriction cleavage in the plasmid in which the mRNA transcript and synthetic fragment would ultmately be joined.

As shown in Fig. 1, the 5' ends of the duplex fragment have single stranded cohesive termini for the Eco RI and Hind III restriction endonucleases to facilitate plasmid construction. The methionine codon at the left end provides a site for initiation of translation. Twelve different oligonucleotides, varying in size from undecamer to hexadecamer, were synthesized by the improved phosphotriester method of Crea, R. Proc. Nat'l. Acad. Sci. USA 75, 5765 (1978). These oligonucleotides, U_1 to U_6 and L_1 to L_6, are indicated by arrows.

10 µg amounts of U_2 through U_6 and L_2 through L_6 were phosphorylated using T_4 polynucleotide kinase and (γ^{32}-P)ATP by a published procedure. Goeddel, D. V. et al. Proc. Nat'l. Acad. Sci. USA 76, 106 (1979).

Three separate T_4 ligase catalyzed reactions were performed: 10 µg of 5'-OH fragment U_1 was combined with the phosphorylated U_2, L_5 and L_6; phosphorylated U_3, U_4, L_3 and L_4 were combined; and 10 µg of 5'-0H fragment L_1 was combined with the phosphorylated L_2, U_5 and U_6. These ligations were carried out at 4°C for 6 hours in 300 µl of 20 mM Tris-HCl (pH 7.5), mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM ATP using 100 units of T_4 ligase. The three ligation mixtures were then combined, 100 units T_4 ligase added, and the reaction allowed to proceed for 12 hours at 20°C. The mixture was ethanol precipitated and electrophoresed on a 10% polyacrylamide gel. The band migrating at 84 bp was sliced from the gel and eluted. pBR322 (1 µg) was treated with Eco RI and Hind III, the large fragment isolated by gel electrophoresis and ligated to the synthetic DNA. This mixture was used to transform E. Coli K-12 strain 294 (end A, thi$^-$, hsr$^-$, hsm$_k^+$). Strain 294 was deposited October 30, 1978 in the American Type Culture Collection (ATCC No. 31446), without restriction.Sequence analysis by the Maxam and Gilbert technique, supra, on the Eco RI - Hind III insert from a plasmid pHGH3 of one transformant confirmed that depicted in Figure 1.

3. Construction of Plasmid for the Bacterial Expression of HGH (Fig. 5)

With the synthetic fragment in pHGH3 and the mRNA transcript in pHGH31, a replicable plasmid containing both fragments was constructed using the expression plasmid pGH6, as shown in Fig. 5. The expression plasmid, which contains tandem lac promoters, was first constructed as follows. A 285 base pair Eco RI fragment containing two 95 base pair UV5 lac promoter fragments separated by a 95 base pair heterlogous DNA fragent was isolated from plasmid pKB268, K. Backman, et al., Cell, Vol. 13, 65-71 (1978). The 285 bp fragment was inserted into the Eco RI site of pBR322 and a clone pGH1 isolated with the promoters oriented toward and in proper reading phase with the gene for tetracycline resistance. The Eco RI site distal to the latter gene was destroyed by partial Eco RI digestion, repair of the resulting single stranded Eco RI ends with DNA polymerase I and recircularization of the plasmid by blunt-end ligation. The resulting plasmid, pGH6, contains a single Eco RI site properly positioned with respect to the promoter system into which the completed gene for HGH could be inserted.

To ready the synthetic fragment for combination with the RNA transcript, 10 µg of pHGH3 was cleaved with Eco RI and Hae III restriction endonucleases and the 77 base pair fragment containing coding sequences for HGH amino acids 1-23 was isolated from an 8% polyacrylamide gel.

The plasmid pHGH 31 (5µg) was next cleaved with Hae III. The 551 bp HGH sequence and a comigrating 540 bp Hae III fragment of pBR322 were purified by gel electrophoresis. Subsequent treatment with Xma I cleaved only the HGH sequence, removing 39 base pairs from the 3' noncoding region. The resulting 512 bp fragment was separated from the 540 bp pBR322 Hae III piece by electrophoresis on a 6% polyacrylamide gel. 0.3 µg of the 77 b.p. Eco RI - Hae III fragment were polymerized with T_4 ligase in a 16 µl reaction for 14 hours at 4°C. The mixture was heated to 70° C for 5' to inactivate the ligase, then treated with Eco RI (to cleave fragments which had dimerized through their Eco RI sites) and with Sma I (to cleave Xma I dimers), yielding a 591 bp fragment with an Eco RI "cohesive" end and a Sma I "blunt" end. After purification on a 6% polyacrylamide gel, approximately 30 ng of this fragment were obtained. It should be noted that the expression plasmid pGH6 contains no Xma I recognition site. However, Sma I recognizes the same site as Xma I, but cuts through the middle of it, yielding blunt ends. The Sma-cleaved terminus of

the fragment derived from gHGH 31 can accordingly be blunt end ligated into pGH6.

The expression plasmid pGH6, containing tandem lac UV5 promoters, was treated successively with Hind III, nuclease S1, and Eco RI and purified by gel electrophoresis. 50 ng of the resulting vector, which had one Eco RI cohesive end and one blunt end was ligated to 10 ng of the 591 bp HGH DNA. The ligation mixture was used to transform E. Coli. X1776. Colonies were selected for growth on tetracycline (12.5 µg/ml). It is noteworthy that insertion of the hybrid HGH gene into pGH6 destroys the promoter for the tetracycline resistance gene, but that the tandem lac promoter permits read-through of the structural gene for tet resistance, retaining this selection characteristic. Approximately 400 transformants were obtained. Filter hybridization by the Grunstein - Hogness procedure, Proc. Nat'l. Acad. Sci. USA, 72, 3961 (1975) identified 12 colonies containing HGH sequences. The plasmids isolated from three of these colonies gave the expected restriction patterns when cleaved with Hae III, Pvu II, and Pst I. The DNA sequence of one clone, pHGH107, was determined.

Human growth hormone expressed by the transformants was easily detected by direct radioimmunoassay performed on serial dilutions of lysed cell supernatants using the Phadebas HGH PRIST kit (Farmacia).

To demonstrate that HGH expression is under the control of the lac promoter, pHGH 107 was transformed into E. Coli strain D1210 a lac+($i^{Q}0+z^{t}y+$), a lac repressor overproducer. Meaningful levels of HGH expression could not be detected until addition of the inducer IPTG (isopropylthiogalactoside).

Removal of the Eco RI site in pHGH107 would leave the ATG start signal the same distance from the ribosome binding site codons of the lac promoter as occurs in nature between those codons and the start signal for β-galactosidase. To determine whether expression would be increased by mimicking this natural spacing we converted pHGH107 to pHGH107-1 by opening the former with Eco RI, digesting the resulting single strand ends with S1 endonuclease, and recircularizing by blunt-end ligation with T4 ligase. Although the resulting plasmid proved likewise capable of expressing HGH, it surprisingly did so to a lesser extent than did pHGH107, as shown by direct radioimmunoassay.

It will be apparent to those skilled in the art that the present invention is not limited to the preferred embodiment just discussed, but rather only to the lawful scope of the appended claims. Variations other than those hitherto discussed will be apparent, whether in the choice of promoter system, parental plasmid, intended polypeptide product or elsewhere. For example, other promoter systems applicable to the present invention include the lambda promoter, the arabinose operon (phi 80 d ara) or the colicine EI, galactose, alkaline phosphatase or tryptophan promoter systems. Host organisms for bacterial expression may be chosen, e.g., from among the Enterobacteriaceae, such as strains of Escherichia coli and Salmonella; Bacillaceae, such as bacillus subtilis; Pneumococcus; Streptococcus; and Haemophilus influenzae. Of course, the choice of organism will control the levels of physical containment in cloning and expression that should be practiced to comply with National Institutes of Health Guidelines for Recombinant DNA, 43 Fed. Reg. 60,080 (1978).

While preferred for bench-scale practice of the present invention, E. Coli. x1776 could prove of limited practicality in large-scale industrial manufacture owing to the debilitations purposefully incorporated in it for biosafety reasons. With appropriate levels of physical, rather than biological, containment such organisms as E. Coli. K-12 strain 294, supra, and E. Coli. strain RR1, genotype: $Pro^{-}Leu^{-}Thi^{-}R_{B^{-}recA+Str^{r}\,Lac\,y^{-}}$ could be employed in larger scale operation. E. Coli. RR1 is derived from E. Coli HB101 (H.W. Boyer, et al, J. Mol.Bio. (1969) 41 459-472) by mating with E. Coli.K12 strain KL16 as the Hfr donor. See J.H. Miller, Experiments in Molecular Genetics (Cold Spring Harbor, New Yor, 1972). A culture of E. Coli. RR1 was deposited October 30, 1978 with the American Type Culture Collection, without restriction as to access (ATCC No. 31343). A culture of x1776 was similarly deposited July 3, 1979 in the American Type Culture Collection (ATCC No. 31537). Deposits of the following were made in the American Type Culture Collection July 3, 1979: plasmid pHGH107 (ATCC No. 40011); plasmid pGH6 (ATCC No. 40012); strain x1776 transformed with pHGH 107 (ATCC No. 31538) and E. Coli K12 strain 294 transformed with pGH6 (ATCC No. 31539).

Organisms produced according to the invention may be employed in industrial scale fermentative production of human growth hormone, yielding product in quantities and for applications hitherto unattainable. For example, transformant E. Coli cultures may be grown up in aqueous media in a steel or other fermentation vessel conventionally aerated and agitated, in aqueous media at, e.g., about 37°C and near neutral pH (e.g., pH 7+ 0.3 supplied with appropriate nutriments such as carbohydrate or glycerol, nitrogen sources such as ammonia sulfate, potassium sources such as potassium phosphate, trace elements, magnesium sulfate and the like. Transformant organisms preferably exhibit one or more selection characteristics, such as antibiotic resistance, so that selection pressures may be imposed to discourage competitive growth of wild-type E. Coli. As an example, in the case of an ampicillin or tetracycline-resistant organism the antibiotic may be added to the fermentation medium to select out wild-type organisms which

lack the resistance characteristic.

Upon completion of fermentation the bacterial suspension is centrifuged or the cellular solids otherwise collected from the broth and then lysed by physical or chemical means. Cellular debris is removed from supernatant and soluble growth hormone isolated and purified.

Human growth hormone may be purified from bacterial extracts using one or a combination of (1) polyethyleneimine fractionation; (2) gel filtration chromatography on Sephacryl S-200; (3) ion exchange chromatography on Biroex-70 resin or CM Sephadex; (4) ammonium sulphate and/or pH fractionation; and (5) affinity chromatography using antibody resins prepared from anti-HGH IgG isolated from immunosensitized animals or hybridomas; and desorbed under acid or slightly denaturing conditions.

Claims

Claims for the following Contracting States : NL, SE

1. A method of constructing a replicable cloning vehicle capable, in a microbial organism, of expressing a particular polypeptide of known amino acid sequence wherein a gene coding for the polypeptide is inserted into a cloning vehicle and placed under the control of an expression promoter,
   characterized in that
   the gene coding for the polypeptide is prepared by
      (a) obtaining by reverse transcription from messenger RNA a first gene fragment for an expression product other than said polypeptide, which fragment comprises at least a substantial portion of the coding sequence for said polypeptide;
      (b) where the first fragment comprises protein-encoding codons for amino acid sequences other than those contained in said polypeptide, eliminating the same while retaining at least a substantial portion of said coding sequence, the resulting fragment nevertheless coding for an expression product other than said polypeptide;
   the product of step (a) or, where required, step (b) being a fragment encoding less than all of the amino acid sequence of said polypeptide;
      (c) providing by organic synthesis one or more gene fragments encoding the remainder of the amino acid sequence of said polypeptide, at least one of said fragments coding for the amino-terminal portion of the polypeptide; and
      (d) deploying the synthetic gene fragment(s) of step (c) and that produced in step (a) or (b), as the case may be, in a replicable cloning vehicle in proper reading phase relative to one another.

2. The method of Claim 1 wherein the cloning vehicle of step (d) is a bacterial plasmid.

3. The method of Claim 2 wherein the synthetic fragment encoding the amino-terminal portion of the polypeptide additionally codes for expression of a specifically cleavable amino acid sequence, and wherein the fragments coding for said polypeptide are deployed downstream from and in reading phase with expressed additionally protein-encoding codons, whereby the conjugated plasmid expression product may be specifically cleaved to yield the polypeptide.

4. The method of Claim 2 wherein the amino acid sequence of the polypeptide is expressable unaccompanied by extraneous conjugated protein.

5. The method of Claim 4 wherein the fragment of step (a) comprises at least a majority of the coding sequence for said polypeptide.

6. The method of Claim 2 wherein a synthetic fragment and a mRNA transcript fragment are ligated to one another before their deployment in the cloning vehicle, and wherein the opposite ends of the fragment and of the transcript are variously single-stranded or blunt so as to ensure ligation of the two fragments in the proper order for expression of said polypeptide.

7. The method of Claim 5 wherein the polypeptide is human growth hormone, and wherein the first fragment comprises protein-encoding codons for amino acid sequences other than those in human growth hormone, and wherein elimination step (b) yields the Hae III restriction enzyme fragment of the first fragment.

8. The method of Claim 7 wherein step (b) includes digestion of the Hae III fragment with a different

restriction enzyme, cleaving away codons for untranslated messenger RNA and simultaneously providing a single-stranded terminus at one end of the resulting fragment.

9. The method of Claim 8 wherein the second restriction enzyme is Xma I.

10. An expression plasmid comprising functional genes for ampicillin and tetracycline resistance and, lying between said genes, a lac promoter system oriented to promote expression of a polypeptide of known amino acid sequence in the direction of the gene for tetracycline resistance and restriction sites being positioned downstream from said promoter
characterized in that
said promoter is a tandem lac promoter and the restriction sites comprise sites, yielding blunt ends upon cleavage, and permitting proper inserting of heterologous DNA, coding for said known amino acid sequence, between said sites so as to come under the control of said promoter system.

11. The plasmid pGH6 (ATCC, No. 40012)
characterized in that
it is obtainable from known pBR322 by inserting a 285 base pair Eco RI fragment known from plasmid pKB268, containing two 95 base pair UV5 lac promoter fragments separated by a 95 base heterologous DNA fragment, into the Eco RI site of pBR322 in proper reading phase with the gene for tetracycline resistance, and destroying the tetracycline gene distal Eco RI site by partial Eco RI digestion, repair of the resulting single-stranded Eco RI ends with DNA polymerase I and recircularization of the plasmid by blunt-end ligation.

12. A replicable bacterial plasmid obtainable according to a method of one of Claims 1 to 9 capable, in a transformant bacterium, of expressing human growth hormone unaccompanied by extraneous conjugated protein, and in which the human growth hormone encoding gene is under the control of tandem lac promoters.

13. A plasmid according to Claim 12 whose human growth hormone encoding DNA comprises in substantial proportion cDNA or a replication thereof.

14. A plasmid according to Claims 12 and 13 which exhibits resistance to at least one antibiotic.

15. A plasmid according to Claim 14 which lacks the tet promoter yet exhibits tetracycline resistance.

16. The plasmid pHGH107 (ATCC, No. 40011),
characterized in that
it is obtainable from plasmid pGH6 (ATCC, No. 40012) by treating pGH6 successively with Hind III, nuclease S1 and Eco RI, and ligating the resulting vector, having one Eco RI cohesive end and one blunt end, with a 591 base pair Eco RI/SmaI fragment of human growth hormone DNA.

17. The plasmid pHGH107-1,
characterized in that
it is obtainable from plasmid pHGH107 (ATCC, No. 40011), by cleaving pHGH107 with Eco RI, digesting the resulting single-strand ends with S1 endonuclease and recirculating by blunt-end ligation with T_4 ligase.

18. A plasmid according to Claim 12 capable in transformant E. coli bacteria, of expressing the DNA sequence coding for the amino acid sequence of human growth hormone.

19. A viable bacterial culture of bacterial transformants comprising plasmids according to any one of Claims 12 to 18.

20. A method of producing human growth hormone in a manner known per se which comprises:
(a) disposing a culture according to Claim 19 within a fermenter vessel comprising aeration and agitation means in an aqueous, nutriment-containing fermentation broth;
(b) growing up the culture under aeration and agitation while supplying additional nutriments as required to maintain vigorous growth;

(c) separating the resulting cellular mass from the fermentation broth;
(d) lysing the cells to free the contents thereof;
(e) separating cellular debris from supernatant; and
(f) isolating and purifying human growth hormone contained in the supernatant.

21. The method of Claim 20 wherein the bacteria are transformant E. coli having a selection characteristic and wherein selection pressure is applied to discourage competition by wild-type E. coli.

22. A plasmid as claimed in Claim 12 which includes two or more of the features specified in Claims 13 to 15 or 18.

23. A method of producing a polypeptide employing the cloning vehicle constructed according to Claim 4 including expressing the amino acid sequences of the polypeptide unaccompanied by extraneous conjugated protein.

24. A method of producing human growth hormone employing the cloning vehicle constructed according to Claim 7 including expressing the amino acid sequence of the human growth hormone free of extraneous N-terminal protein.

Claims for the following Contracting State : AT

1. A method of constructing a replicable cloning vehicle capable, in a microbial organism, of expressing a particular polypeptide of known amino acid sequence wherein a gene coding for the polypeptide is inserted into a cloning vehicle and placed under the control of an expression promoter,
characterized in that
the gene coding for the polypeptide is prepared by
(a) obtaining by reverse transcription from messenger RNA a first gene fragment for an expression product other than said polypeptide, which fragment comprises at least a substantial portion of the coding sequence for said polypeptide;
(b) where the first fragment comprises protein-encoding codons for amino acid sequences other than those contained in said polypeptide, eliminating the same while retaining at least a substantial portion of said coding sequence, the resulting fragment nevertheless coding for an expression product other than said polypeptide;
the product of step (a) or, where required, step (b) being a fragment encoding less than all of the amino acid sequence of said polypeptide;
(c) providing by organic synthesis one or more gene fragments encoding the remainder of the amino acid sequence of said polypeptide, at least one of said fragments coding for the amino-terminal portion of the polypeptide; and
(d) deploying the snythetic gene fragment(s) of step (c) and that produced in step (a) or (b), as the case may be, in a replicable cloning vehicle in proper reading phase relative to one another.

2. The method of Claim 1 wherein the cloning vehicle of step (d) is a bacterial plasmid.

3. The method Claim 2 wherein the synthetic fragment encoding the amino-terminal portion of the polypeptide additionally codes for expression of a specifically cleavable amino acid sequence, and wherein the fragments coding for said polypeptide are deployed downstream from and in reading phase with expressed additionally protein-encoding codons, whereby the conjugated plasmid expression product may be specifically cleaved to yield the polypeptide.

4. The method of Claim 2 wherein the amino acid sequence of the polypeptide is expressable unaccompanied by extraneous conjugated protein.

5. The method of Claim 4 wherein the fragment of step (a) comprises at least a majority of the coding sequence for said polypeptide.

6. The method of Claim 2 wherein a synthetic fragment and a mRNA transcript fragment are ligated to one another before their deployment in the cloning vehicle, and wherein the opposite ends of the fragment and of the transcript are variously single-stranded or blunt so as to ensure ligation of the two

fragments in the proper order for expression of said polypeptide.

7. The method of Claim 5 wherein the polypeptide is human growth hormone, and wherein the first fragment comprises protein-encoding codons for amino acid sequences other than those in human growth hormone, and wherein elimination step (b) yields the Hae III restriction enzyme fragment of the first fragment.

8. The method Claim 7 wherein step (b) includes digestion of the Hae III fragment with a different restriction enzyme, cleaving away codons for untranslated messenger RNA and simultaneously providing a single-stranded terminus at one end of the resulting fragment.

9. The method of Claim 8 wherein the second restriction enzyme is Xma I.

10. A method for preparing an expression plasmid comprising functional genes for ampicillin and tetracycline resistance and, lying between said genes, a tandem lac promoter system oriented to promote expression of a polypeptide of known amino acid sequence in the direction of the gene for tetracycline resistance, plural restriction sites being positioned downstream from said promoter system which yield, respectively, cohesive and blunt ends upon cleavage, permitting proper inserting of heterologous DNA, coding for said known amino acid sequence, between said sites so as to come under the control of said promoter system, by inserting a tandem lac promoter into suited plasmids by a manner known per se.

11. A method for preparing the plasmid pGH6 (ATCC, No. 40012) by inserting into known pBR322 a 285 base pair Eco RI fragment from known plasmid pKB268, containing two 95 base pair UV5 lac promoter fragments separated by a 95 base pair heterologous DNA fragment, into the Eco RI site of pBR322 in proper reading phase with the gene for tetracycline resistance, and destroying the tetracycline gene distal Eco RI site by partial Eco RI digestion, repair of the resulting single-stranded Eco RI ends with DNA polymerase I and recircularization of the plasmid by blunt-end ligation.

12. A method for preparing a replicable bacterial plasmid according to one of Claims 1 to 9, the bacterial plasmid being capable of expressing human growth hormone unaccompanied by extraneous conjugated protein, and in which the human growth hormone encoding gene is under the control of tandem lac promoters.

13. A method according to Claim 12,
characterized in that
the human growth hormone encoding DNA comprises in substantial proportion cDNA or a replication thereof.

14. A method according to Claims 12 or 13,
characterized in that
the plasmid exhibits resistance to at least one antibiotic.

15. A method according to Claim 14,
characterized in that
the plasmid lacks the tet promoter yet exhibits tetracycline resistance.

16. A method for preparing plasmid pHGH107 (ATCC, No. 40011) by treating plasmid pGH6 (ATCC, No. 40012) successively with Hind III, nuclease S1 and Eco RI, and ligating the resulting vector, having one Eco RI cohesive end and one blunt end, with a 591 base pair Eco RI/SmaI fragment of human growth hormone DNA.

17. A method for preparing plasmid pHGH107-1 by cleaving plasmid pHGH107 (ATCC, No. 40011) with Eco RI, digesting the resulting single-strand ends with SI endonuclease and recirculating by blunt-end ligation with T_4 ligase.

18. A method for preparing a plasmid according to Claim 12,
characterized in that

the plasmid is capable in transformant E. coli bacteria, of expressing the DNA sequence coding for the amino acid sequence of human growth hormone.

19. A viable bacterial culture of bacterial transformants comprising plasmids prepared according to any one of Claims 12 to 18.

20. A method of producing human growth hormone in a manner known per se which comprises:
(a) disposing a culture according to Claim 19 within a fermenter vessel comprising aeration and agitation means in an aqueous, nutriment-containing fermentation broth;
(b) growing up the culture under aeration and agitation while supplying additional nutriments as required to maintain vigorous growth;
(c) separating the resulting cellular mass from the fermentation broth;
(d) lysing the cells to free the contents thereof;
(e) separating cellular debris from supernatant; and
(f) isolating and purifying human growth hormone contained in the supernatant.

21. The method of Claim 20 wherein the bacteria are transformant E. coli having a selection characteristic and wherein selection pressure is applied to discourage competition by wild-type E. coli.

22. A method according to Claim 12,
characterized in that
the plasmid includes two or more of the features specified in Claims 13 to 15 or 18.

23. A method of producing a polypeptide employing the cloning vehicle constructed according to Claim 4 including expressing the amino acid sequences of the polypeptide unaccompanied by extraneous conjugated protein.

24. A method of producing human growth hormone employing the cloning vehicle constructed according to Claim 7 including expressing the amino acid sequence of the human growth hormone free of extraneous N-terminal protein.

Patentansprüche
Patentansprüche für folgende Vertragsstaaten : NL, SE

1. Verfahren zum Konstruieren eines replizierbaren Klonierungsvektors, der die Fähigkeit hat, in einem mikrobiellen Organismus ein bestimmtes Polypeptid mit bekannter Aminosäuresequenz zu exprimieren, wobei ein für das Polypeptid codierendes Gen in einen Klonierungsvektor eingebaut und unter die Kontrolle eines Expressionspromotors gestellt wird,
dadurch gekennzeichnet, daß
das für das Polypeptid codierende Gen hergestellt wird durch
(a) Erhalten eines ersten Genfragments für ein von dem Polypeptid verschiedenes Expressionsprodukt durch Reverse Transkription von Messenger-RNA, wobei das Fragment mindestens einen wesentlichen Teil der kodierenden Sequenz für das Polypeptid umfaßt;
(b) soweit das erste Fragment proteincodierende Codons für andere Aminosäuresequenzen als die in dem Polypeptid enthaltenen umfaßt, das Eliminieren dieser unter Beibehaltung von mindestens einem wesentlichen Teil der codierenden Sequenz, wobei das resultierende Fragment nichtsdestoweniger für ein anderes Expressionsprodukt als das Polypeptid codiert;
wobei das Produkt von Schritt (a) oder, soweit erforderlich, Schritt (b) ein Fragment ist, das für weniger als die gesamte Aminosäuresequenz des Polypeptids codiert;
(c) Bereitstellen eines oder mehrerer Genfragmente, die für den Rest der Aminosäuresequenz des Polypeptids codieren, durch organische Synthese, wobei mindestens eines der Fragmente für den Aminoende-Teil des Polypeptids codiert; und
(d) Anordnen des synthetischen Genfragments (der synthetischen Genfragmente) von Schritt (c) und des je nach dem in Schritt (a) oder (b) produzierten in einem replizierbaren Klonierungsvektor in dem richtigen Leseraster relativ zu einander.

2. Verfahren nach Anspruch 1, worin der Klonierungsvektor von Schritt (d) ein bakterielles Plasmid ist.

3. Verfahren nach Anspruch 2, worin das synthetische Fragment, welches für den Aminoendeteil des Polypeptids codiert, zusätzlich für die Expression einer spezifisch spaltbaren Aminosäuresequenz codiert, und worin die für das Polypeptid codierenden Fragmente stromabwärts von und im Leseraster mit exprimierten, zusätzlich für Protein codierenden Codons angeordnet sind, wobei das konjugierte Produkt der Expression des Plasmids spezifisch gespalten werden kann, um das Polypeptid zu ergeben.

4. Verfahren nach Anspruch 2, worin die Aminosäuresequenz des Polypeptids ohne anhängendes konjugiertes fremdes Protein exprimierbar ist.

5. Verfahren nach Anspruch 4, worin das Fragment von Schritt (a) mindestens den größeren Teil der codierenden Sequenz für das Polypeptid umfaßt.

6. Verfahren nach Anspruch 2, worin ein synthetisches Fragment und ein mRNA-Transkriptionsfragment vor ihrer Einbringung in den Klonierungsvektor miteinander verknüpft werden, und worin die entgegengesetzten Enden des Fragments und des Transkriptionsfragments abwechselnd einzelsträngig oder mit glatten Enden versehen sind, so daß die Verknüpfung der beiden Fragmente in der richtigen Reihenfolge zur Expression des Polypeptids sichergestellt ist.

7. Verfahren nach Anspruch 5, worin das Polypeptid menschliches Wachstumshormon ist, und worin das erste Fragment Protein-codierende Codons für von den Aminosäuresequenzen in menschlichem Wachstumshormon verschiedene Aminosäuresequenzen umfaßt, und worin der Eliminierungsschritt (b) das Hae III-Restriktionsenzymfragment des ersten Fragments ergibt.

8. Verfahren nach Anspruch 7, worin der Schritt (b) die Spaltung des Hae III-Fragments mit einem anderen Restriktionsenzym einschließt, wobei Codons für nicht-translatierte Messenger-RNA abgeschnitten werden und gleichzeitig ein Einzelstrang-Ende an einem Ende des resultierenden Fragments bereitgestellt wird.

9. Verfahren nach Anspruch 8, worin das zweite Restriktionsenzym Xma I ist.

10. Expressionsplasmid, welches funktionelle Gene für Ampicillin- und Tetracyclinresistenz und ein zwischen diesen Genen liegendes lac-Promotorsystem, welches so angeordnet ist, daß es die Expression eines Polypeptids von bekannter Aminosäuresequenz in der Richtung des Gens für Tetracyclinresistenz anregt, und
stromabwärts von diesem Promotor liegende Restriktionsstellen umfaßt,
dadurch gekennzeichnet, daß
dieser Promotor ein lac-Tandem-Promotor ist und die Restriktionsstellen Stellen umfassen, die glatte Enden beim Spalten ergeben und das richtige Einbauen von heterologer DNA, die für die bekannte Aminosäuresequenz codiert, zwischen diesen Stellen ermöglichen, so daß diese unter die Kontrolle des Promotorsystems kommt.

11. Plasmid pGH6 (ATCC, Nr. 40012)
dadurch gekennzeichnet, daß
es aus dem bekannten pBR322 erhalten werden kann durch das Einbauen eines 285 Basenpaare großen Eco-RI-Fragments, das von Plasmid pKB268 her bekannt ist, welches zwei 95 Basenpaare große UV5-lac-Promotorfragmente enthält, die durch ein 95 Basen großes heterologes DNA-Fragment getrennt sind, in die Eco-RI-Stelle von pBR322 in dem richtigen Leseraster mit dem Gen für Tetracyclinresistenz, und das Zerstören der zum Tetracyclin-Gen distalen Eco-RI-Stelle durch teilweise Spaltung mit Eco-RI, Reparatur der resultierenden einzelsträngigen Eco-RI-Enden mit DNA-Polymerase I und Wiederherstellung des Plasmidrings durch Verknüpfung der glatten Enden.

12. Replizierbares bakterielles Plasmid, welches gemäß einem Verfahren nach einem der Ansprüche 1 bis 9 erhalten werden kann, welches die Fähigkeit hat, in einem transformierten Bakterium menschliches Wachstumshormon ohne anhängendes konjugiertes fremdes Protein zu exprimieren, und in dem das Gen, welches für das menschliche Wachstumshormon codiert, unter der Kontrolle von lac-Tandem-Promotoren steht.

13. Plasmid nach Anspruch 12, dessen für menschliches Wachstumshormon codierende DNA einen wesentlichen Anteil cDNA oder eine Replikation dieser umfaßt.

14. Plasmid nach Anspruch 12 und 13, welches Resistenz gegen mindestens ein Antibiotikum aufweist.

15. Plasmid nach Anspruch 14, welchem der tet-Promotor fehlt und welches trotzdem Tetracyclinresistenz aufweist.

16. Plasmid pHGH107 (ATCC, Nr. 40011),
dadurch gekennzeichnet, daß
es aus Plasmid pGH6 (ATCC, Nr. 40012) erhalten werden kann durch aufeinanderfolgendes Behandeln von pGH6 mit Hind III, Nuklease S1 und Eco RI und Verknüpfen des resultierenden Vektors, welcher ein Eco RI-kohäsives Ende und ein glattes Ende hat, mit einem 591 Basenpaare großen Eco RI/SmaI-Fragment der DNA des menschlichen Wachstumshormons.

17. Plasmid pHGH107-1,
dadurch gekennzeichnet, daß
es aus Plasmid pHGH107 (ATCC, Nr. 40011) erhalten werden kann durch Spalten von pHGH107 mit Eco RI, Spalten der resultierenden einzelsträngigen Enden mit S1-Endonuklease und Wiederherstellung des Rings durch Verknüpfung der glatten Enden mit T_4-Ligase.

18. Plasmid nach Anspruch 12, welches die Fähigkeit hat, in transformierten E. coli Bakterien die DNA-Sequenz, die für die Aminosäuresequenz des menschlichen Wachstumshormons codiert, zu exprimieren.

19. Lebensfähige Bakterienkultur von bakteriellen Transformanten, die Plasmide nach einem der Ansprüche 12 bis 18 umfaßt.

20. Verfahren zum Herstellen von menschlichem Wachstumshormon auf eine an sich bekannte Weise, welches umfaßt:
(a) Einbringen einer Kultur nach Anspruch 19 in einen Fermentationsbehälter, der Belüftungs- und Durchmischungsanlagen in einer wässrigen, nährstoffhaltigen Fermentationsbrühe umfaßt;
(b) Hochwachsen der Kultur mit Belüftung und Durchmischen unter Zufuhr zusätzlicher Nährstoffe, wie sie zur Aufrechterhaltung eines kräftigen Wachstums erforderlich sind;
(c) Trennen der resultierenden zellulären Masse von der Fermentationsbrühe;
(d) Auflösen der Zellen zum Freisetzen ihres Inhalts;
(e) Trennen der zellulären Bruchstücke vom Überstand; und
(f) Isolieren und Reinigen von im Überstand enthaltenem menschlichen Wachstumshormon.

21. Verfahren nach Anspruch 20, worin die Bakterien transformierte E. coli sind, die ein Selektionsmerkmal haben und worin Selektionsdruck ausgeübt wird, um die Konkurrenz durch Wildtyp-E. coli zu unterdrükken.

22. Plasmid nach Anspruch 12, welches zwei oder mehr der in den Ansprüchen 13 bis 15 oder 18 spezifizierten Merkmale einschließt.

23. Verfahren zum Herstellen eines Polypeptids unter Verwendung des nach Anspruch 4 konstruierten Klonierungsvektors, welches das Exprimieren der Aminosäuresequenzen des Polypeptids ohne anhängendes konjugiertes fremdes Protein einschließt.

24. Verfahren zum Herstellen von menschlichem Wachstumshormon unter Verwendung des nach Anspruch 7 konstruierten Klonierungsvektors, welches das Exprimieren der Aminosäuresequenzen des menschlichen Wachstumshormons, das frei von fremden N-terminalen Protein ist, einschließt.

Patentansprüche für folgenden Vertragsstaat : AT

1. Verfahren zum Konstruieren eines replizierbaren Klonierungsvektors, der die Fähigkeit hat, in einem mikrobiellen Organismus ein bestimmtes Polypeptid mit bekannter Aminosäuresequenz zu exprimieren,

worin ein für das Polypeptid codierendes Gen in einen Klonierungsvektor eingebaut und unter die Kontrolle eines Expressionspromotors gestellt wird,

dadurch gekennzeichnet, daß

das für das Polypeptid codierende Gen hergestellt wird durch

(a) Erhalten eines ersten Genfragments für ein von dem Polypeptid verschiedenes Expressionsprodukt durch Reverse Transkription von Messenger-RNA, wobei das Fragment mindestens einen wesentlichen Teil der codierenden Sequenz für das Polypeptid umfaßt;

(b) soweit das erste Fragment proteincodierende Codons für andere Aminosäuresequenzen als die in dem Polypeptid enthaltenen umfaßt, das Eliminieren dieser unter Beibehaltung von mindestens einem wesentlichen Teil der codierenden Sequenz, wobei das resultierende Fragment nichtsdestoweniger für ein anderes Expressionsprodukt als das Polypeptid codiert;

wobei das Produkt von Schritt (a) oder, soweit erforderlich, Schritt (b) ein Fragment ist, das für weniger als die gesamte Aminosäuresequenz des Polypeptids codiert;

(c) Bereitstellen eines oder mehrerer Genfragmente, die für den Rest der Aminosäuresequenz des Polypeptids codieren, durch organische Synthese, wobei mindestens eines der Fragmente für den Aminoende-Teil des Polypeptids codiert; und

(d) Anordnen des synthetischen Genfragments (der synthetischen Genfragmente) von Schritt (c) und des je nach dem in Schritt (a) oder (b) produzierten in einem replizierbaren Klonierungsvektor in dem richtigen Leseraster relativ zu einander.

2. Verfahren nach Anspruch 1, worin der Klonierungsvektor von Schritt (d) ein bakterielles Plasmid ist.

3. Verfahren nach Anspruch 2, worin das synthetische Fragment, welches für den Aminoende-Teil des Polypeptids codiert, zusätzlich für die Expression einer spezifisch spaltbaren Aminosäuresequenz codiert, und worin die für das Polypeptid codierenden Fragmente stromabwärts von und im Leseraster mit exprimierten, zusätzlich für Protein codierenden Codons angeordnet sind, wobei das konjugierte Produkt der Expression des Plasmids spezifisch gespalten werden kann, um das Polypeptid zu ergeben.

4. Verfahren nach Anspruch 2, worin die Aminosäuresequenz des Polypeptids ohne anhängendes konjugiertes fremdes Protein exprimierbar ist.

5. Verfahren nach Anspruch 4, worin das Fragment von Schritt (a) mindestens den größeren Teil der codierenden Sequenz für das Polypeptid umfaßt.

6. Verfahren nach Anspruch 2, worin ein synthetisches Fragment und ein mRNA-Transkriptionsfragment vor ihrer Einbringung in den Klonierungsvektor miteinander verknüpft werden, und worin die entgegengesetzten Enden des Fragments und des Transkriptionsfragments abwechselnd einzelsträngig oder mit glatten Enden versehen sind, so daß die Verknüpfung der beiden Fragmente in der richtigen Reihenfolge zur Expression des Polypeptids sichergestellt ist.

7. Verfahren nach Anspruch 5, worin das Polypeptid menschliches Wachstumshormon ist, und worin das erste Fragment Protein-codierende Codons für von den Aminosäuresequenzen in menschlichem Wachstumshormon verschiedene Aminosäuresequenzen umfaßt, und worin der Eliminierungsschritt (b) das Hae III-Restriktionsenzymfragment des ersten Fragments ergibt.

8. Verfahren nach Anspruch 7, worin der Schritt (b) die Spaltung des Hae III-Fragments mit einem anderen Restriktionsenzym einschließt, wobei Codons für nicht-translatierte Messenger-RNA abgeschnitten werden und gleichzeitig ein Einzelstrang-Ende an einem Ende des resultierenden Fragments bereitgestellt wird.

9. Verfahren nach Anspruch 8, worin das zweite Restriktionsenzym Xma I ist.

10. Verfahren zum Herstellen eines Expressionsplasmids, welches funktionelle Gene für Ampicillin- und Tetracyclinresistenz und ein zwischen diesen Genen liegendes lac-Tandem-Promotorsystem, welches so angeordnet ist, daß es die Expression eines Polypeptids von bekannter Aminosäuresequenz in der

Richtung des Gens für Tetracyclinresistenz anregt, umfaßt, wobei mehrere Restriktionsstellen stromabwärts von diesem Promotorsystem gelegen sind, die bei Spaltung kohäsive bzw. glatte Enden ergeben, welche das richtige Einbauen von heterologer DNA, die für die bekannte Aminosäuresequenz codiert, zwischen diese Stellen ermöglichen, so daß sie unter die Kontrolle des Promotorsystems kommt, durch das Einbauen eines lac-Tandem-Promotors in geeignete Plasmide auf eine an sich bekannte Weise.

11. Verfahren zum Herstellen des Plasmids pGH6 (ATCC, Nr. 40012) durch das Einbauen eines 285 Basenpaare großen Eco RI-Fragments aus dem bekannten Plasmid pKB268 , welches zwei 95 Basenpaare große UV5-lac-Promotorfragmente enthält, die durch ein 95 Basen großes heterologes DNA-Fragment getrennt sind, in das bekannte pBR322, in die Eco RI-Stelle von pBR322 in dem richtigen Leseraster mit dem Gen für Tetracyclinresistenz, und das Zerstören der zum Tetracyclin-Gen distalen Eco RI-Stelle durch teilweise Spaltung mit Eco RI, Reparatur der resultierenden einzelsträngigen Eco RI-Enden mit DNA-Polymerase I und Wiederherstellung des Plasmidrings durch Verknüpfung der glatten Enden.

12. Verfahren zum Herstellen eines replizierbaren bakteriellen Plasmids nach einem der Ansprüche 1 bis 9, welches die Fähigkeit hat, menschliches Wachstumshormon ohne anhängendes konjugiertes fremdes Protein zu exprimieren, und in dem das Gen, welches für das menschliche Wachstumshormon codiert, unter der Kontrolle von lac-Tandem-Promotoren steht.

13. Verfahren nach Anspruch 12,

dadurch gekennzeichnet, daß

die für menschliches Wachstumshormon codierende DNA einen wesentlichen Anteil cDNA oder eine Replikation dieser umfaßt.

14. Verfahren nach Anspruch 12 oder 13,

dadurch gekennzeichnet, daß
das Plasmid Resistenz gegen mindestens ein Antibiotikum aufweist.

15. Verfahren nach Anspruch 14,

dadurch gekennzeichnet, daß

dem Plasmid der tet-Promotor fehlt und es trotzdem Tetracyclinresistenz aufweist.

16. Verfahren zum Herstellen von Plasmid pHGH107 (ATCC, Nr. 40011) durch aufeinanderfolgendes Behandeln des Plasmids pGH6 (ATCC, Nr. 40012) mit Hind III, Nuklease S1 und Eco RI und Verknüpfen des resultierenden Vektors, welcher ein Eco RI-kohäsives Ende und ein glattes Ende hat, mit einem 591 Basenpaare großen Eco RI/SmaI-Fragment der DNA des menschlichen Wachstumshormons.

17. Verfahren zum Herstellen von Plasmid pHGH107-1, durch Spalten von Plasmid pHGH107 (ATCC, Nr. 40011) mit Eco RI, Spalten der resultierenden einzelsträngigen Enden mit S1-Endonuklease und Wiederherstellung des Rings durch Verknüpfung der glatten Enden mit T_4-Ligase.

18. Verfahren zum Herstellen eines Plasmids nach Anspruch 12,

dadurch gekennzeichnet, daß

das Plasmid die Fähigkeit hat, in transformierten E. coli Bakterien die DNA-Sequenz, die für die Aminosäuresequenz des menschlichen Wachstumshormons codiert, zu exprimieren.

19. Lebensfähige Bakterienkultur von bakteriellen Transformanten, die nach einem der Ansprüche 12 bis 18 hergestellte Plasmide umfaßt.

20. Verfahren zum Herstellen von menschlichem Wachstumshormon auf eine an sich bekannte Weise, welches umfaßt:
(a) Einbringen einer Kultur nach Anspruch 19 in einen Fermentationsbehälter, der Belüftungs- und Durchmischungsanlagen in einer wässrigen, nährstoffhaltigen Fermentationsbrühe umfaßt;
(b) Hochwachsen der Kultur mit Belüftung und Durchmischen unter Zufuhr zusätzlicher Nährstoffe, wie sie zur Aufrechterhaltung eines kräftigen Wachstums erforderlich sind;
(c) Trennen der resultierenden zellulären Masse von der Fermentationsbrühe;
(d) Auflösen der Zellen zum Freisetzen ihres Inhalts;
(e) Trennen der zellulären Bruchstücke vom Überstand; und
(f) Isolieren und Reinigen von im Überstand enthaltenem menschlichen Wachstumshormon.

21. Verfahren nach Anspruch 20, worin die Bakterien transformierte E. coli sind, die ein Selektionsmerkmal haben und worin Selektionsdruck ausgeübt wird, um die Konkurrenz durch Wildtyp-E. coli zu unterdrükken.

22. Verfahren nach Anspruch 12,

dadurch gekennzeichnet, daß

das Plasmid zwei oder mehr der in den Ansprüchen 13 bis 15 oder 18 spezifizierten Merkmale einschließt.

23. Verfahren zum Herstellen eines Polypeptids unter Verwendung des nach Anspruch 4 konstruierten Klonierungsvektors, welches das Exprimieren der Aminosäuresequenzen des Polypeptids ohne anhängendes konjugiertes fremdes Protein einschließt.

24. Verfahren zum Herstellen von menschlichem Wachstumshormon unter Verwendung des nach Anspruch 7 konstruierten Klonierungsvektors, welches das Exprimieren der Aminosäuresequenzen des menschlichen Wachstumshormons, das frei von fremden N-terminalen Protein ist, einschließt.

Revendications
Revendications pour les Etats contractants suivants : NL, SE

1. Procédé de construction d'un véhicule de clonage réplicable, capable d'exprimer, dans un organisme microbien, un polypeptide particulier ayant une séquence d'amino-acides connue, selon lequel un gène codant pour le polypeptide est inséré dans un véhicule de clonage et mis sous le contrôle d'un promoteur d'expression ;
caractérisé en ce que
le gène codant pour le peptide est préparé :
(a) en produisant par transcription inverse à partir d'un ARN messager, un premier fragment de gène codant pour un produit d'expression autre que ledit polypeptide, ce fragment comprenant au moins une partie importante de la séquence codant pour ledit polypeptide ;
(b) lorsque le premier fragment comprend des codons codant pour une protéine dont les séquences d'amino-acides sont différentes de celles contenues dans ledit polypeptide, en éliminant ces derniers et en conservant au moins une partie importante de cette séquence codante, le fragment résultant codant néanmoins pour un produit d'expression autre que ledit polypeptide ;
le produit de l'étape (a) ou, lorsque cela est nécessaire, de l'étape (b) consistant en un fragment codant pour moins de la totalité de la séquence d'amino-acides dudit polypeptide ;
(c) en produisant par synthèse organique, un ou plusieurs fragments de gène codant pour le reste de la séquence d'amino-acides dudit polypeptide, au moins un de ces fragments codant pour la partie amino-terminale du polypeptide ; et
(d) en introduisant le ou les fragments de gène synthétiques produits dans l'étape (c) et ceux produits dans l'étape (a) ou (b), ainsi que cela peut être le cas, dans un véhicule de clonage réplicable selon une phase de lecture appropriée les uns par rapport aux autres.

2. Procédé selon la revendication 1, dans lequel le véhicule de clonage de l'étape (d) est un plasmide bactérien.

3. Procédé selon la revendication 2, dans lequel le fragment synthétique codant pour la partie amino-terminale du polypeptide, code en outre pour l'expression d'une séquence d'amino-acides susceptible d'être coupée de manière spécifique, et dans lequel les fragments codants pour ledit polypeptide sont introduits en aval et en phase de lecture par rapport aux codons codant pour l'expression d'une protéine supplémentaire, de sorte que le produit d'expression conjugué du plasmide, peut être coupé de manière spécifique pour procurer le polypeptide.

4. Procédé selon la revendication 2, dans lequel la séquence d'amino-acides du polypeptide, peut être exprimée sans protéine étrangère conjuguée.

5. Procédé selon la revendication 4, dans lequel le fragment de l'étape (a) comprend au moins une majeure partie de la séquence codant pour ledit polypeptide.

6. Procédé selon la revendication 2, dans lequel le fragment synthétique et un fragment de transcription d'ARNm sont liés l'un à l'autre avant leur introduction dans le véhicule de clonage, et dans lequel les extrémités opposées du fragment et du produit de transcription, sont diversement mono-caténaires ou franches, de façon à assurer la liaison des deux fragments dans l'ordre approprié pour l'expression dudit polypeptide.

7. Procédé selon la revendication 5, dans lequel le polypeptide est une hormone de croissance humaine, et dans lequel le premier fragment comprend des codons codant pour une protéine ayant des séquences d'amino-acides autres que celles présentes dans l'hormone de croissance humaine, et dans lequel l'étape d'élimination (b) procure le fragment comportant un site de restriction par l'enzyme Hae III, dérivé du premier fragment.

8. Procédé selon la revendication 7, dans lequel l'étape (b) comprend la digestion du fragment Hae III avec une enzyme de restriction différente, l'élimination par coupure des codons correspondant à un ARN messager non traduit, et la formation simultanée d'une terminaison mono-caténaire à une extrémité du fragment résultant.

9. Procédé selon la revendication 8, dans lequel la deuxième enzyme de restriction est Xma I.

10. Plasmide d'expression comprenant des gènes fonctionnels de résistance à l'ampicilline et à la tétracycline entre lesquels s'étend un système promoteur lac orienté de façon à favoriser l'expression d'un polypeptide ayant une séquence d'amino-acides connue, dans la direction du gène de résistance à la tétracycline, et des sites de restriction étant disposés en aval de ce promoteur, caractérisé en ce que ledit promoteur est un promoteur lac en tandem et en ce que les sites de restriction comprennent des sites procurant par coupure, des extrémités franches, et permettant l'insertion appropriée d'un ADN hétérologue codant pour ladite séquence d'amino-acides connue, entre ces sites, de façon à être contrôlés par ce système promoteur.

11. Plasmide pGH6 (ATCC, N° 40012) caractérisé en ce qu'il est obtenu à partir du plasmide pBR322 connu, par insertion d'un fragment Eco Ri de 285 paires de bases dérivé du plasmide pKB268 connu, contenant deux fragments de promoteur lac UV5 de 95 paires de bases séparés par un fragment d'ADN hétérologue de 95 paires de bases dans le site Eco RI de pBR322 selon une phase de lecture appropriée par rapport au gène de résistance à la tétracycline, et par destruction du site Eco RI distal du gène de résistance à la tétracycline par digestion partielle avec Eco RI, en réparant les extrémités Eco RI mono-caténaires résultantes avec l'ADN polymérase I, et en recircularisant le plasmide par liaison d'extrémités franches.

12. Plasmide bactérien réplicable obtenu selon la méthode d'une des revendications 1 à 9, capable d'exprimer dans une bactérie transformée, une hormone de croissance humaine sans protéine étrangère conjuguée, et dans lequel le gène codant pour l'hormone de croissance humaine, est contrôlé par les promoteurs lac en tandem.

13. Plasmide selon la revendication 12, dont l'ADN codant pour l'hormone de croissance humaine comprend une proportion importante d'ADNc ou d'un produit de réplication de celui-ci.

14. Plasmide selon la revendication 12 ou 13, qui est résistant à au moins un antibiotique.

15. Plasmide selon la revendication 14, qui ne comprend pas le promoteur tet et est encore résistant à la tétracycline.

16. Plasmide pHGH107 (ATCC, N° 40011) caractérisé en ce qu'il est obtenu à partir du plasmide pGH6 (ATCC, N° 40012), par traitements successifs du pGH6, avec Hind III, la nucléase S1 et Eco RI, et en liant le vecteur résultant comportant une extrémité Eco RI cohésive et une extrémité franche, avec un fragment Eco RI/SmaI de 591 paires de bases dérivé de l'ADN d'hormone de croissance humaine.

17. Plasmide pHGH107-1 (ATCC, N° 40011) caractérisé en ce qu'il est obtenu à partir du plasmide pHGH107 (ATCC, N° 40011), par coupure du plasmide pHGH107 avec Eco RI, par digestion des extrémités mono-caténaires résultantes avec l'endonucléase SI, et par recircularisation par liaison d'extrémités franches avec la ligase T_4.

18. Plasmide selon la revendication 12, capable d'exprimer, dans des bactéries de E. coli transformées, la séquence d'ADN codant pour la séquence d'amino-acides de l'hormone de croissance humaine.

19. Culture bactérienne viable de transformants bactériens, comprenant des plasmides selon l'une quelconque des revendications 12 à 18.

20. Procédé de production d'une hormone de croissance humaine de façon connue en soi, selon lequel :
(a) on prépare une culture selon la revendication 19 dans un fermenteur comprenant des moyens d'aération et d'agitation, dans un bouillon de fermentation aqueux contenant des nutriments ;
(b) on cultive la culture sous aération et agitation, en fournissant des nutriments supplémentaires nécessaires pour maintenir une croissance vigoureuse ;
(c) on sépare la masse cellulaire résultante du bouillon de fermentation ;
(d) on lyse les cellules pour libérer leur contenu ;
(e) on sépare les débris cellulaires du surnageant ; et
(f) on isole et on purifie l'hormone de croissance humaine contenue dans le surnageant ;

21. Procédé selon la revendication 20, dans lequel les bactéries consistent en des cellules de E. coli transformées ayant une caractéristique de sélection, et dans lequel on applique une pression de sélection pour empêcher la compétition avec E. coli de type sauvage.

22. Plasmide selon la revendication 12, possédant au moins deux des caractéristiques mentionnées dans les revendications 13 à 15 ou 18.

23. Procédé de production d'un polypeptide mettant en oeuvre le véhicule de clonage construit selon la revendication 4, selon lequel on exprime les séquences d'amino-acides du polypeptide, sans protéine étrangère conjuguée.

24. Procédé de production d'une hormone de croissance humaine mettant en oeuvre le véhicule de clonage construit la revendication 7, selon lequel on exprime la séquence d'amino-acides de l'hormone de croissance humaine, sans protéine étrangère N-terminale.

Revendications pour l'Etat contractant suivant : AT

1. Procédé de construction d'un véhicule de clonage réplicable, capable d'exprimer, dans un organisme microbien, un polypeptide particulier ayant une séquence d'amino-acides connue, selon lequel un gène codant pour le polypeptide est inséré dans un véhicule de clonage et mis sous le contrôle d'un promoteur d'expression ;
caractérisé en ce que
le gène codant pour le peptide est préparé :
(a) en produisant par transcription inverse à partir d'un ARN messager, un premier fragment de gène codant pour un produit d'expression autre que ledit polypeptide, ce fragment comprenant au moins une partie importante de la séquence codant pour ledit polypeptide ;
(b) lorsque le premier fragment comprend des codons codant pour une protéine dont les séquences

d'amino-acides sont différentes de celles contenues dans ledit polypeptide, en éliminant ces derniers et en conservant au moins une partie importante de cette séquence codante, le fragment résultant codant néanmoins pour un produit d'expression autre que ledit polypeptide ;

le produit de l'étape (a) ou, lorsque cela est nécessaire, de l'étape (b) consistant en un fragment codant pour moins de la totalité de la séquence d'amino-acides dudit polypeptide ;

(c) en produisant par synthèse organique, un ou plusieurs fragments de gène codant pour le reste de la séquence d'amino-acides dudit polypeptide, au moins un de ces fragments codant pour la partie amino-terminale du polypeptide ; et

(d) en introduisant le ou les fragments de gène synthétiques produits dans l'étape (c) et ceux produits dans l'étape (a) ou (b), ainsi que cela peut être le cas, dans un véhicule de clonage réplicable selon une phase de lecture appropriée les uns par rapport aux autres.

2. Procédé selon la revendication 1, dans lequel le véhicule de clonage de l'étape (d) est un plasmide bactérien.

3. Procédé selon la revendication 2, dans lequel le fragment synthétique codant pour la partie amino-terminale du polypeptide, code en outre pour l'expression d'une séquence d'amino-acides susceptible d'être coupée de manière spécifique, et dans lequel les fragments codants pour ledit polypeptide sont introduits en aval et en phase de lecture par rapport aux codons codant pour l'expression d'une protéine supplémentaire, de sorte que le produit d'expression conjugué du plasmide, peut être coupé de manière spécifique pour procurer le polypeptide.

4. Procédé selon la revendication 2, dans lequel la séquence d'amino-acides du polypeptide, peut être exprimée sans protéine étrangère conjuguée.

5. Procédé selon la revendication 4, dans lequel le fragment de l'étape (a) comprend au moins une majeure partie de la séquence codant pour ledit polypeptide.

6. Procédé selon la revendication 2, dans lequel le fragment synthétique et un fragment de transcription d'ARNm sont liés l'un à l'autre avant leur introduction dans le véhicule de clonage, et dans lequel les extrémités opposées du fragment et du produit de transcription, sont diversement mono-caténaires ou franches, de façon à assurer la liaison des deux fragments dans l'ordre approprié pour l'expression dudit polypeptide.

7. Procédé selon la revendication 5, dans lequel le polypeptide est une hormone de croissance humaine, et dans lequel le premier fragment comprend des codons codant pour une protéine ayant des séquences d'amino-acides autres que celles présentes dans l'hormone de croissance humaine, et dans lequel l'étape d'élimination (b) procure le fragment comportant un site de restriction par l'enzyme Hae III, dérivé du premier fragment.

8. Procédé selon la revendication 7, dans lequel l'étape (b) comprend la digestion du fragment Hae III avec une enzyme de restriction différente, l'élimination par coupure des codons correspondant à un ARN messager non traduit, et la formation simultanée d'une terminaison mono-caténaire à une extrémité du fragment résultant.

9. Procédé selon la revendication 8, dans lequel la deuxième enzyme de restriction est Xma I.

10. Procédé de préparation d'un plasmide d'expression comprenant des gènes fonctionnels de résistance à l'ampicilline et à la tétracycline entre lesquels s'étend un système promoteur lac en tandem orienté de façon à favoriser l'expression d'un polypeptide ayant une séquence d'amino-acides connue, dans la direction du gène de résistance à la tétracycline, plusieurs sites de restriction étant disposés en aval de ce système promoteur, qui procurent respectivement par coupure, des extrémités cohésives et franches, permettant l'insertion appropriée d'un ADN hétérologue codant pour ladite séquence d'amino-acides connue entre ces sites, de façon à être contrôlés par ce système promoteur, par insertion d'un promoteur lac en tandem dans des plasmides appropriés, de façon connue en soi.

11. Procédé de préparation du plasmide pGH6 (ATCC, N° 40012) par insertion dans le plasmide pBR322 connu, d'un fragment Eco Ri de 285 paires de bases dérivé du plasmide pKB268 connu, contenant

deux fragments de promoteur lac UV5 de 95 paires de bases séparés par un fragment d'ADN hétérologue de 95 paires de bases dans le site Eco RI de pBR322 selon une phase de lecture appropriée par rapport au gène de résistance à la tétracycline, et par destruction du site Eco RI distal du gène de résistance à la tétracycline par digestion partielle avec Eco RI, en réparant les extrémités Eco RI mono-caténaires résultantes avec l'ADN polymérase I, et en recircularisant le plasmide par liaison d'extrémités franches.

12. Procédé de préparation d'un plasmide bactérien réplicable selon l'une des revendications 1 à 9, le plasmide bactérien étant capable d'exprimer une hormone de croissance humaine sans protéine étrangère conjuguée, et dans lequel le gène codant pour l'hormone de croissance humaine, est contrôlé par les promoteurs lac en tandem.

13. Procédé selon la revendication 12, caractérisé en ce que l'ADN codant pour l'hormone de croissance humaine comprend une proportion importante d'ADNc ou d'un produit de réplication de celui-ci.

14. Procédé selon la revendication 12 ou 13, caractérisé en ce que le plasmide est résistant à au moins un antibiotique.

15. Procédé selon la revendication 14, caractérisé en ce que le plasmide ne comprend pas le promoteur tet et est encore résistant à la tétracycline.

16. Procédé de préparation du plasmide pHGH107 (ATCC, N° 40011) par traitements successifs du plasmide pGH6 (ATCC, N° 40012), avec Hind III, la nucléase S1 et Eco RI, et en liant le vecteur résultant comportant une extrémité Eco RI cohésive et une extrémité franche, avec un fragment Eco RI/SmaI de 591 paires de bases dérivé de l'ADN d'hormone de croissance humaine.

17. Procédé de préparation du plasmide pHGH107-1 par coupure du plasmide pHGH107 (ATCC, N° 40011) avec Eco RI, digestion des extrémités mono-caténaires résultantes avec l'endonucléase SI, et recircularisation par liaison d'extrémités franches avec la ligase T_4.

18. Procédé de préparation d'un plasmide selon la revendication 12, caractérisé en ce que le plasmide est capable d'exprimer, dans des bactéries de E. coli transformées, la séquence d'ADN codant pour la séquence d'amino-acides de l'hormone de croissance humaine.

19. Culture bactérienne viable de transformants bactériens, comprenant des plasmides préparés selon l'une quelconque des revendications 12 à 18.

20. Procédé de production d'une hormone de croissance humaine de façon connue en soi, selon lequel :
(a) on prépare une culture selon la revendication 19 dans un fermenteur comprenant des moyens d'aération et d'agitation, dans un bouillon de fermentation aqueux contenant des nutriments ;
(b) on cultive la culture sous aération et agitation, en fournissant des nutriments supplémentaires nécessaires pour maintenir une croissance vigoureuse ;
(c) on sépare la masse cellulaire résultante du bouillon de fermentation ;
(d) on lyse les cellules pour libérer leur contenu ;
(e) on sépare les débris cellulaires du surnageant ; et
(f) on isole et on purifie l'hormone de croissance humaine contenue dans le surnageant ;

21. Procédé selon la revendication 20, dans lequel les bactéries consistent en des cellules de E. coli transformées ayant une caractéristique de sélection, et dans lequel on applique une pression de sélection pour empêcher la compétition avec E. coli de type sauvage.

22. Procédé selon la revendication 12, caractérisé en ce que le plasmide possède au moins deux des caractéristiques mentionnées dans les revendications 13 à 15 ou 18.

23. Procédé de production d'un polypeptide mettant en oeuvre le véhicule de clonage construit selon la revendication 4, selon lequel on exprime les séquences d'amino-acides du polypeptide, sans protéine étrangère conjuguée.

24. Procédé de production d'une hormone de croissance humaine mettant en oeuvre le véhicule de clonage construit la revendication 7, selon lequel on exprime la séquence d'amino-acides de l'hormone de croissance humaine, sans protéine étrangère N-terminale.

HGH (1-24)

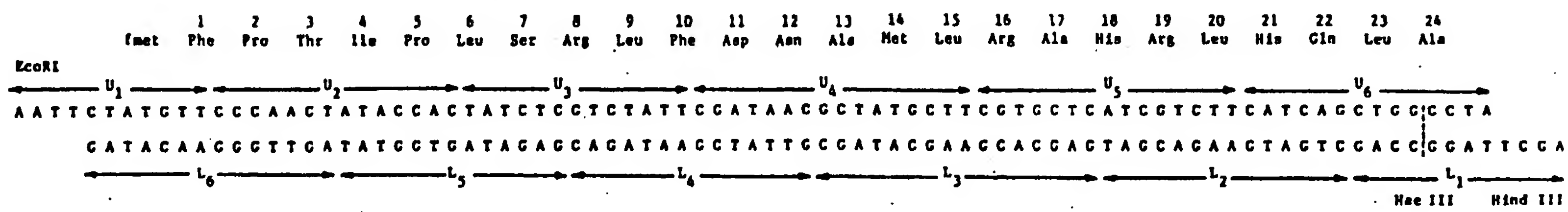

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
fmet Phe Pro Thr Ile Pro Leu Ser Arg Leu Phe Asp Asn Ala Met Leu Arg Ala His Arg Leu His Gln Leu Ala
EcoRI
U1 U2 U3 U4 U5 U6
A A T T C T A T G T T C C C A A C T A T A C C A C T A T C T C C T C T A T T C C A T A A C G C T A T G C T T C G T G C T C A T C G T C T T C A T C A G C T G G C C T A
G A T A C A A G G G T T G A T A T G C T G A T A G A G C A G A T A A G C T A T T G C G A T A C G A A G C A C G A G T A G C A G A A G T A G T C G A C C G G A T T C G A
L6 L5 L4 L3 L2 L1
Hae III
Hind III

FIG. 1.

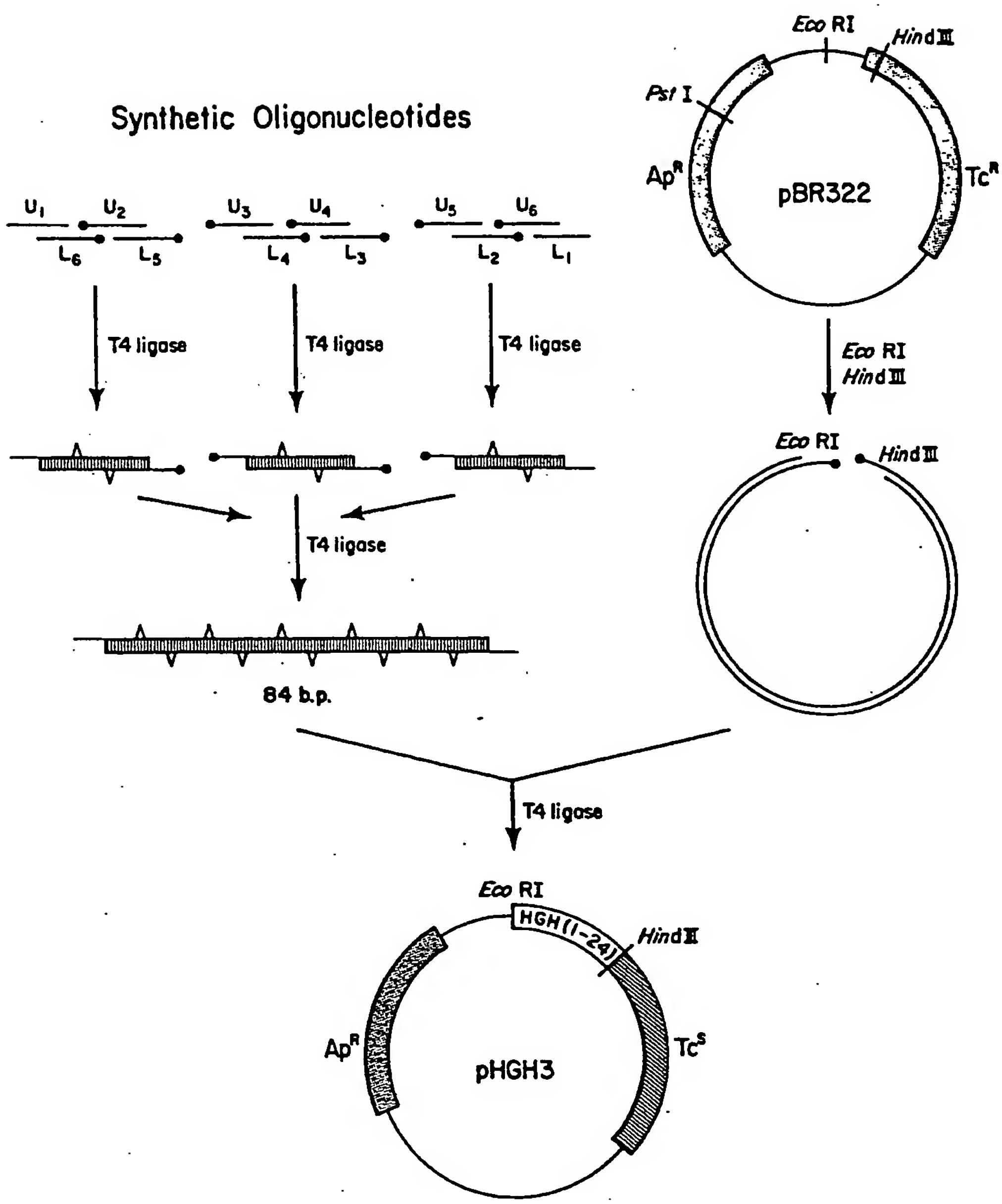

Synthetic Oligonucleotides
U1
U2
U3
U4
U5
U6
L6
L5
L4
L3
L2
L1
T4 ligase
T4 ligase
T4 ligase
T4 ligase
84 b.p.
Eco RI
Hind III
Pst I
Ap^R
pBR322
Tc^R
Eco RI
Hind III
Eco RI
Hind III
T4 ligase
Eco RI
HGH(1-24)
Hind III
Ap^R
pHGH3
Tc^S

FIG. 2.

```
24                                                   40
Ala Phe Asp Thr Tyr Gln Glu Phe Glu Glu Ala Tyr Ile Pro Lys Glu Gln Lys Tyr Ser Phe Leu Gln Asn Pro Gln Thr Ser
5'...G GCC UUU GAC ACC UAC CAG GAG UUU GAA GAA GCC UAU AUC CCA AAG GAA CAG AAG UAU UCA UUC CUG CAG AAC CCC CAG ACC UCC
                                                                                       Pst I

                    60                                                                                  80
Leu Cys Phe Ser Glu Ser Ile Pro Thr Pro Ser Asn Arg Glu Glu Thr Gln Gln Lys Ser Asn Leu Glu Leu Leu Arg Ile Ser Leu Leu
CUC UGU UUC UCA GAG UCU AUU CCG ACA CCC UCC AAC AGG GAG GAA ACA CAA CAG AAA UCC AAC CUA GAG CUG CUC CGC AUC UCC CUG CUG

                                                  100
Leu Ile Gln Ser Trp Leu Glu Pro Val Gln Phe Leu Arg Ser Val Phe Ala Asn Ser Leu Val Tyr Gly Ala Ser Asp Ser Asn Val Tyr
CUC AUC CAG UCG UGG CUG GAG CCC GUG CAG UUC CUC AGG AGU GUC UUC GCC AAC AGC CUA GUG UAC GGC GCC UCU GAC AGC AAC GUC UAU

                                          120                                                                    140
Asp Leu Leu Lys Asp Leu Glu Glu Gly Ile Gln Thr Leu Met Gly Arg Leu Glu Asp Gly Ser Pro Arg Thr Gly Gln Ile Phe Lys Gln
GAC CUC CUA AAG GAC CUA GAG GAA GGC AUC CAA ACG CUG AUG GGG AGG CUG GAA GAU GGC AGC CCC CGG ACU GGG CAG AUC UUC AAG CAG
                                                                                                  Bgl II
                                                  160
Thr Tyr Ser Lys Phe Asp Thr Asn Ser His Asn Asp Asp Ala Leu Leu Lys Asn Tyr Gly Leu Leu Tyr Cys Phe Arg Lys Asp Met Asp
ACC UAC AGC AAG UUC GAC ACA AAC UCA CAC AAC GAU GAC GCA CUA CUC AAG AAC UAC GGG CUG CUC UAC UGC UUC AGG AAG GAC AUG GAC

                    180                                     191
Lys Val Glu Thr Phe Leu Arg Ile Val Gln Cys Arg Ser Val Glu Gly Ser Cys Gly Phe Stop
AAG GUC GAG ACA UUC CUG CGC AUC GUG CAG UGC CGC UCU GUG GAG GGC AGC UGU GGC UUC UAG CUGCCCGGGUGGCAUCCCUGUGACCCCUCCCCAGU
                                                          Pvu II                  Sma I
                                                                                  Xma I
GCCUCUCCUGGCC....3'
     Hae III
```

FIG. 3.

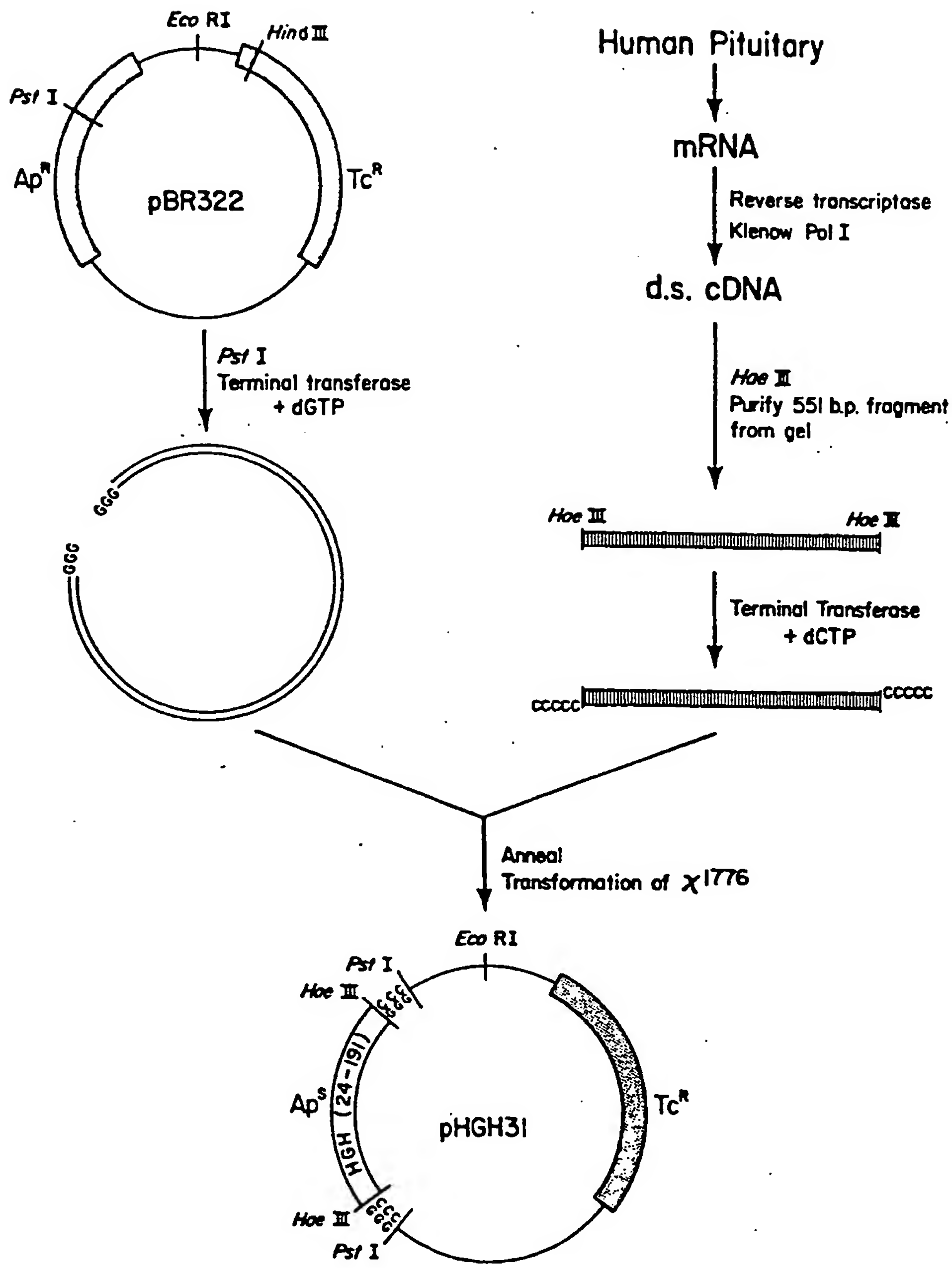

Eco RI
Hind III
Pst I
Ap^R
pBR322
Tc^R
Pst I
Terminal transferase
+ dGTP
GGG
GGG
Human Pituitary
mRNA
Reverse transcriptase
Klenow Pol I
d.s. cDNA
Hae III
Purify 551 b.p. fragment
from gel
Hae III
Hae III
Terminal Transferase
+ dCTP
CCCCC
CCCCC
Anneal
Transformation of χ1776
Eco RI
Pst I
Hae III
Ap^S
HGH (24-191)
pHGH31
Tc^R
Hae III
Pst I

FIG. 4.

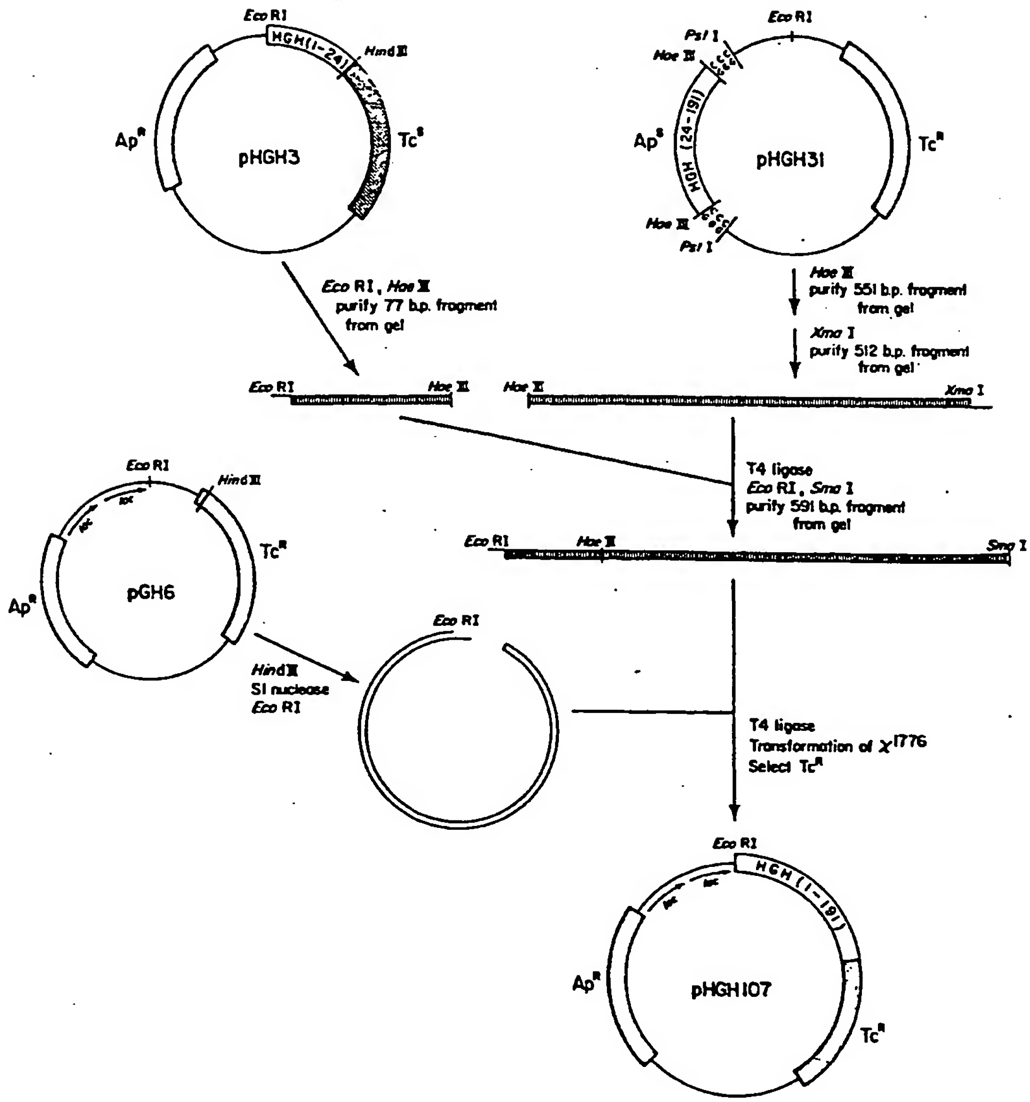

Eco RI
HGH(1-24)
Hind III
Ap^R
pHGH3
Tc^S
Eco RI
Pst I
Hae III
HGH (24-191)
Ap^S
pHGH31
Tc^R
Hae III
Pst I
Eco RI, Hae III
purify 77 b.p. fragment
from gel
Hae III
purify 551 b.p. fragment
from gel
Xma I
purify 512 b.p. fragment
from gel
Eco RI
Hae III
Hae III
Xma I
Eco RI
Hind III
Tc^R
pGH6
Ap^R
T4 ligase
Eco RI, Sma I
purify 591 b.p. fragment
from gel
Eco RI
Hae III
Sma I
Eco RI
Hind III
SI nuclease
Eco RI
T4 ligase
Transformation of χ1776
Select Tc^R
Eco RI
HGH (1-191)
Ap^R
pHGH107
Tc^R

FIG. 5.